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THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

Woodland Avenue and Thirty-sixth Street, Philadelphia, Pa.

IRON UTILIZATION IN DOGS ON MILK DIETS [1]

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(Received for publication December 11, 1939)

ONE FIGURE

Whole milk, unlike other diets which have been used in iron metabolism studies with dogs (Fontes and Thivolle, '36; Huebner and Frerichs, '37; Hahn et al., '39), is relatively low in iron. When proper precautions against iron contamination are taken, the amount of iron supplied in milk as the sole article of diet, is insignificant. In the course of our investigation it was determined that growing dogs consume about 140 cc. of whole milk per kilogram of body weight per day, and mature dogs slightly less. Iron analyses made on samples of milk taken at various times indicated that the iron intake due to the milk feeding was only about 1 mg. per dog per week.

When Potter, Elvehjem and Hart ('38) began their study of anemia in dogs on milk diets, they used 30 mg. of iron daily, a figure based on the earlier work of Schultze, Elvehjem and Hart ('36 a, '36 b) with pigs. Since calculation of the data of Potter et al. ('38) showed that less than one-fourth of the iron fed at the 30 mg. level was used to build blood hemoglobin (Hb), we concluded that the level was probably higher than was actually needed and that some of it was either stored or excreted. In order to study iron utilization more exactly it was important to know an approximate mini-

[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Works Progress Administration.

mal figure for the amount of iron which would allow both optimal growth and blood formation. In order to arrive at such a figure one must consider not only the amount of iron in blood hemoglobin in the dog at any definite time, but also the amount of iron which would be present in the dog's body as muscle hemoglobin iron and parenchyma iron. The following considerations were of value in this regard.

According to Hahn ('37), iron is distributed in the body of the normal dog in the following approximate percentages of the total: blood Hb iron 60%; muscle Hb iron 6%; parenchyma iron 15%; and storage iron 20%. The experimental data of Whipple ('26) and Hahn and Whipple ('36) indicate that the variation from these values is low in the cases of blood Hb iron and parenchyma iron, but may be high for muscle Hb iron and storage iron. If 30 mg. of dietary iron are completely absorbed and distributed as above in a dog's body, there will result about 5.3 gm. of blood Hb (0.1 mg. Fe $\simeq$ 0.294 gm. Hb). The daily blood Hb production of a puppy growing at the rate of 100 gm. per day and maintaining a blood Hb level of 15 gm. per 100 cc. of blood, however, is only about 1.2 gm. The dose of 30 mg. of iron is therefore theoretically many times higher than that needed by growing dogs of average sizes (adult weight 12 to 16 kg.). Even when the added requirement for iron in puppies severely anemic at weaning is calculated, this level is found to be many times too high.

In all calculations the blood was considered to be 8% of the body weight. Although the validity of this assumption is always open to question, consideration of the data will show that any probable error, due to unaccounted-for changes in blood volume, would hardly be so great as to change its significance.

In our studies the primary objective was to determine the effect of copper deficiency on the extent of iron utilization. We ('39), had already produced a copper deficiency of such severity that no response was shown to iron alone. Incidentally, during the course of the experiments evidence was obtained substantiating our earlier finding that cobalt in very small amounts may stimulate hematopoiesis.

EXPERIMENTAL

Dogs placed on raised screens in wooden cages at 4 weeks of age and fed whole milk only became severely anemic (Hb 3.5 to 6.0 gm. per 100 cc. of blood) in 3 to 9 weeks. At this point either 10 mg. of iron as $FeCl_3$ alone, or 10 mg. of iron plus 2 mg. of copper as $CuSO_4$ was fed. Manganese as a solution of $MnCl_2$ was fed at a level of 1 mg. per day to all dogs.

Two litters of four dogs each were made anemic as above and used in experiments to establish the following points: (1) How soon would a copper deficiency affect the rate of hemoglobin formation in dogs fed iron alone? (2) What level of iron would just meet the needs of the growing anemic dogs?

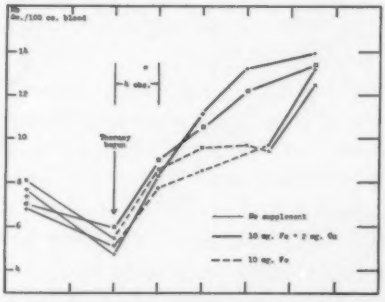

Fig. 1 Hemoglobin curves of four puppies allowed to become anemic on milk alone and then fed iron or iron plus copper.

Dogs fed iron alone for a period and finally a supplement of copper. Responses in four dogs fed 10 mg. of iron alone per day were typified by increases in blood Hb to a level of 8 to 10 gm. per 100 cc. of blood during the first week or two of therapy, after which no further increase occurred until copper was introduced (fig. 1). Calculations indicated that 80 to 90% of the iron ingested during the first week of therapy was converted to blood Hb. In succeeding weeks, however, almost none of the iron fed was returned as blood Hb. When copper was finally given to dogs which had ceased building Hb, there occurred an early resumption of the curve upward to normal levels. In all of these cases the amount of iron

ingested was considerably in excess of the amount theoretically needed. Thus it appeared that a copper deficiency became manifest within a week or two after the beginning of iron therapy. Figure 1 shows the typical Hb curves of some of the dogs fed iron alone and iron plus copper.

Dogs fed iron plus copper from the beginning of the test. Theoretically 10 mg. of iron per day will answer the needs of growing anemic dogs and allow some storage. Also, in the presence of adequate copper, this amount of iron would be expected to allow a maximal rate of hemoglobin regeneration. Each of four dogs receiving 10 mg. of iron and 2 mg. of copper daily showed rapid increases in blood Hb level during the first weeks of therapy. After the Hb level of the blood had reached 10 gm. per 100 cc., increases were much less rapid. In all cases the Hb level and the weight increases were greater than those shown by the four dogs receiving iron alone. Although calculated iron utilization approached 100% during the first few weeks of therapy, the per cent of utilization decreased rapidly thereafter. Thus iron utilization did not parallel iron ingestion and after 6 to 8 weeks of therapy only 60 to 70% of the total iron ingested could be accounted for. The most likely explanation seemed to be that the level of iron fed was in excess of the capacity of the dogs to utilize it and that the excess iron must have been either stored or excreted. The second possibility, that the small amounts of iron involved would not be excreted but would be efficiently stored appeared likely, and the following experiment was designed from this viewpoint.

Experiments with measured iron intake. The iron intake was limited to only that amount which would be expected to meet the needs of the animal for growth and Hb building and all of this iron was supplied during the first few weeks of therapy. The data obtained on two closely matched litter mate puppies are reported with all essential details in tables 1 and 2.

The per cent of iron appearing as blood hemoglobin iron in dogs 19 and 20 is in fair agreement with the figure of 60% found analytically by Hahn and Whipple ('36). The somewhat

higher values for our dogs may be accounted for by the lack of any storage iron.

Although dog no. 20, which did not receive copper during the early growth stage, showed the same per cent conversion of its iron to blood hemoglobin iron as did dog no. 19 which received copper, it should be borne in mind that dog no. 20 received only one-half as much iron as did dog no. 19 and maintained a consistently lower hemoglobin level in the blood.

TABLE 1

Analysis of iron utilization in dog receiving a limited amount of iron together with copper during the early growth stage

DOG NO. 19	IRON UTILIZED DURING PERIOD %
A. Five weeks following weaning, dog was fed the milk diet + 10 mg. Fe and 2 mg. Cu daily. Essential data obtained are indicated below:	
1. Weight at start of iron therapy = 3070 gm. Level of Hb in the blood = 3.6 gm. per 100 cc. Hb in dog at start $= \frac{0.08 \times 3070 \times 3.6}{100} = 9$ gm.	
2. Iron fed during 13-week period = 910 mg. equivalent to 268 gm. Hb (1 mg. Fe ≎ 0.294 gm. Hb) Total body Hb at end of period $= \frac{0.08 \times 12400 \times 14.47}{100} = 143$ gm. Hb made during period = 143 − 9 = 134 gm. Conversion of 910 mg. Fe to blood Hb $= \frac{100 \times 134 \text{ gm. Hb (actual)}}{268 \text{ gm. Hb (theoretical)}} =$	50
B. Had any of the above iron been stored? Dog was again fed milk diet alone, and bled:	
1. 920 cc. of blood ≎ 111 gm. Hb removed in following 8-week period. Total body Hb at end of period $= \frac{0.08 \times 13300 \times 7.28}{100} = 77$ gm. Hb made during period = 77 − (143 − 111) = 45 gm. Total Hb made in 21 weeks = 134 + 45 = 179 gm. Hb Conversion of 910 mg. Fe to blood Hb $= \frac{100 \times 179 \text{ gm. (actual)}}{268 \text{ gm. (theoretical)}} =$	67
2. Further bleeding resulted in loss of Hb, indicating depletion of iron stores.	
3. Estimated total parenchyma Fe =	15
Estimated total muscle Hb Fe =	6
Estimated storage Fe =	0
4. Total iron accounted for =	88

Dog no. 19 received 2 mg. of Cu per day during the 13 weeks of therapy and 1 mg. of Mn per day throughout the experiment.

TABLE 2

Analysis of iron utilization in dog receiving a limited amount of iron but no copper during the early growth stage

DOG NO. 20. LITTERMATE TO DOG NO. 19

IRON UTILIZED DURING PERIOD %

A. Fe fed alone with milk to deplete Cu stores:

1. Weight at start of Fe therapy = 2250 gm.
   Level of Hb in the blood = 4.6 gm. per 100 cc.
   Hb in dog at start = $\frac{0.08 \times 2250 \times 4.6}{100} = 8$ gm.
2. Fe fed during first 3 weeks of 23-week period = 460 mg. ≎ 135 gm. Hb.
   Total body Hb at end of period = $\frac{0.08 \times 10900 \times 10.97}{100} = 95$ gm.
   Hb made during period = 95 — 8 = 87 gm.
   Conversion of 460 mg. Fe to blood Hb = $\frac{100 \times 87 \text{ gm. Hb (actual)}}{135 \text{ gm. Hb (theoretical)}} = 64$

B. Bleeding to induce use of any Fe stored during previous period:
   Dog maintained on milk alone during period

1. 370 cc. of blood ≎ 37 gm. Hb removed 3-week period
   Total body Hb at end of period = $\frac{0.08 \times 9600 \times 8.6}{100} = 66$ gm.
   Hb made during period = 66 — (95 — 37) = 8 gm.
   Total Hb made during 26 weeks = 86 + 8 = 94 gm.
   Conversion of 460 mg. Fe to blood Hb = $\frac{100 \times 94}{135}$ = ... 70
   The appearance of the dog at this stage was one of acute nutritional deficiency and the Cu level of the blood was only 30 to 40 mcg. per 100 cc.
2. Further bleeding resulted in a proportionate loss of Hb.

C. Period of iron therapy:

1. Fe fed during next 5-week period = 700 mg. ≎ 206 gm. Hb.
   Conversion of Fe fed during period C to blood Hb = $\frac{100 \times 6 \text{ gm. Hb (actual)}}{206 \text{ gm. Hb (theoretical)}}$ 3
   Blood Hb remained at 8.5 gm. per 100 cc. and the dog remained apathetic.

D. Period of Fe + Cu therapy:

1. Cu introduced at a level of 2 mg. per day for 3 weeks.
   At end of 8-week period 1120 mg. Fe fed ≎ 329 gm. Hb.
   Conversion of Fe fed during periods C and D to blood Hb = $\frac{100 \times 47}{329}$ = ... 14
   Conversion of Fe fed during period D to blood Hb = $\frac{100 \times 41}{124}$ = .. 33
   Blood Hb level = 11 gm. per 100 cc.
   General condition markedly improved. Hair coat became darker.

E. Period of Fe + Cu + Co therapy:

1. Co introduced at level of 0.5 mg. per day for 3 weeks
   At end of 11-week period 1540 mg. Fe fed ≎ 453 gm. Hb.
   Conversion of Fe fed during periods C, D, and E to blood Hb = $\frac{100 \times 99}{453}$ = ... 22
   Conversion of Fe fed during period E to blood Hb = $\frac{100 \times 52}{124}$ = ..42

Also dog no. 20 grew at a much slower rate than dog no. 19 and showed many effects of the copper deficiency, such as poor coat, frequent anorexia, and lack of body fat. The skeletal growth of the two dogs, however, was practically identical.

DISCUSSION

The individual phases of iron metabolism such as absorption, transport, storage and utilization are probably all conditioned to a large extent by the nutritive state of the animal. The extremely efficient use of small amounts of dietary iron shown by our dogs on milk diets suggests that under these conditions all phases of iron metabolism proceed in excellent harmony. Although copper did not appear to be essential to absorption or storage of iron, it was shown to be absolutely essential to utilization of iron to build hemoglobin.

Hutchison ('38) clearly showed recently that copper enhances the ability of the human infant to utilize stored iron. Barer and Fowler ('37), Maurer, Greengard, Curtis and Kluver ('34), and Josephs ('39) have demonstrated the effect of copper in increasing utilization of iron in human infants. Josephs ('39) recently drew attention to the phenomenon previously observed by Reimann, Fritsch and Schick ('37), and Brock ('37) that the hemoglobin response often ceases in anemic infants even though sufficient iron has been given to warrant an increase to 100%. In view of our work we would expect this to occur if copper were not supplied with iron. Josephs further points out that "in the first 6 months of life when the conditions are such that relatively rapid hemoglobin formation takes place, iron of the stores is thoroughly utilized." This may be explained by the fact that the period of early infancy is characterized by both high copper and high iron stores and that copper depletion, or dilution in the tissues, must parallel that of iron. Frost, Potter, Elvehjem and Hart ('40) have recently shown the need for copper with iron in overcoming hemorrhagic anemia in dogs on milk diets. The addition of copper wherever iron therapy is indicated seems no less logical to us than the addi-

tion of the iron itself since only in the presence of adequate copper is iron useful.

In their recent studies using radio-active iron, Hahn, Bale, Lawrence and Whipple ('39) obtained evidence showing that the extent of iron absorption is proportional to the need for it. Our work lends strong support to this idea and further shows that iron absorption and utilization may approach 100% under well-defined conditions of need. We cannot refrain from again pointing out that the ration used by these workers, as they have so often demonstrated, does not provide their experimental animals with optimal amounts of those many factors which are so clearly needed to allow an optimal rate of blood building.

Some degree of caution should be urged regarding the use of cobalt as a hematopoietic agent. Although many instances in which small amounts of cobalt have appeared to stimulate hematopoiesis in dogs on milk diets are on record in this laboratory, a clear-cut need for this element has not yet been established. We have found it actually to inhibit hematopoiesis under certain conditions of anemia (Frost, Elvehjem and Hart, '39) and have also succeeded in producing polycythemia under still different conditions. Experiments on the need for cobalt and its polycythemia producing action are now being made.

The very small amount of dietary iron needed by anemic puppies on milk diets both to overcome their anemia and to grow normally is noteworthy. This is especially true when one considers the massive doses of iron used in therapy of so-called iron-deficiency anemia in human infants. That large amounts of iron are more effective than small doses in curing anemia has been recognized for some time (Heath, '33; Witts, '33), but why this should be the case is not at all apparent. In this regard the importance of the general nutritive state cannot be over emphasized. It is readily apparent that infants subsisting largely on milk are prone to develop not only a deficiency of iron, but of copper as well. Another case in hand which suggests the inadequacy of iron alone in treatment of all secondary anemias is that of vitamin B_6 anemia in dogs

(Fouts, Helmer, Lepkovsky and Jukes, '38). McKibbin, Black, Madden and Elvehjem ('39) have produced severe anemia in dogs by the use of a vitamin B_6 deficient ration which supplies adequate iron. The anemia was cured by addition of the crystalline vitamin alone.

Although Hahn ('37) and Heath and Patek ('37) in their recent reviews on iron metabolism minimize the importance of copper as an adjunct to iron in hematopoiesis, they offer no workable solution to the question of massive iron dosage. Other deficiencies than that of copper may operate to prevent or hinder utilization of dietary iron, but the probability of copper deficiency as the limiting factor in many cases of hypochromic anemia still demands clinical attention. Consideration of all the factors clearly involved is most certainly required in any investigation or treatment of anemia.

CONCLUSIONS

1. Ten milligrams of soluble inorganic iron per dog per day are somewhat in excess of the amount needed by dogs growing at the rate of 100 gm. per day. Two milligrams of copper per day proved adequate. The ratio of requirement of iron to copper is only about 5 to 1.

2. Even when supplied in small amounts, iron may be stored. This stored iron becomes available for hemoglobin building only in the presence of adequate copper.

3. The per cent of iron utilization for blood hemoglobin building will depend upon the dietary supply of all factors concerned in hematopoietic activity. When only the theoretical requirement of iron is fed to dogs on iron-free, but otherwise adequate diets, utilization of that iron will approach 100%.

4. Further evidence has been obtained for the importance of cobalt as an adjunct to copper and iron for maximal hematopoiesis in dogs on milk diets.

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THE ANTI GREY HAIR VITAMIN, A NEW FACTOR IN THE VITAMIN B COMPLEX [1]

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SEVEN FIGURES

(Received for publication November 14, 1939)

When black and piebald rats were fed several weeks on certain artificial diets deficient in certain factors of the vitamin B complex, we observed that the black hair turned grey (Lunde and Kringstad, '38). This phenomenon was especially pronounced with diets rich in vitamin B_6 and poor in the so-called "filtrate factor." This greying of the fur of black rats was also observed some years ago by Bakke, Aschehoug and Zbinden ('30) when using a certain vitamin B-free diet and wheat germ as a source of vitamin B. These authors were of the opinion that this phenomenon was caused by the presence of some poisonous compound in the wheat germ rather than by the lack of some new factor of the vitamin B complex. Morgan, Cook and Davison ('38) simultaneously with us announced that they had observed greying of black rats on diets poor in the filtrate factor. These authors were of the opinion that the greying of the fur was due to lack of the "filtrate factor." Our experiments, however, indicate that there is a new vitamin in the B complex distinct from the growth-promoting factor present in the "filtrate" fraction.

[1] Read at the 98th Meeting of the American Chemical Society in Boston, Mass., September, 1939.

[2] Research Laboratory of the Norwegian Canning Industry.

It is generally recognized that the vitamin B complex contains B_1 or thiamin, B_2 or riboflavin, nicotinic acid or pellagra-preventive factor, B_6 recently demonstrated to be 2-methyl-3-hydroxy-4,5-di(hydroxymethyl) pyridine, "filtrate factor" or chick antidermatitis substance which appears to be identical with pantothenic acid, and the rat growth "factor W" of Elvehjem and associates (Elvehjem, Koehn and Oleson, '36; Frost and Elvehjem, '37, '39; Oleson, Bird et al., '39) which may be separated from the "filtrate factor" by precipitation with alcohol and ether. Several authors have used the term "filtrate factor" to mean the growth-promoting factor or factors remaining in the solution after treatment with fuller's earth intended to remove B_6. This, however, is rather unfortunate, because the filtrate probably contains several distinct factors. For example, the chick anti-dermatitis factor (pantothenic acid) is present as well as the rat growth factor W. According to Woolley et al. ('38), this chick antidermatitis factor can be extracted with ether in neutral solution. We have been able to show that the rat growth factor contained in the filtrate after treatment of a yeast extract with Fuller's earth is not extracted with ether under these conditions (Kringstad and Lunde, '39). This indicates that our rat growth factor is distinct from the chick antidermatitis factor. In order to define it more clearly, we prefer to designate the rat growth factor in the filtrate as B_w.

GROWTH-PROMOTING FILTRATE FACTOR B_w

When an extract of fish liver is treated with fuller's earth, vitamin B_6 is adsorbed. We have been able to show that the filtrate thus freed from B_6 has growth-promoting properties when given to rats subsisting on a diet containing sufficient amounts of thiamin, riboflavin, nicotinic acid and B_6 (Lunde and Kringstad, '38). This result is in accordance with the observations made by Lepkovsky, Jukes and Krause ('36) working with liver, and by Edgar and Macrae ('37) working with yeast. These results, however, have not always been verified by other investigators. We have found that this may

be due to the fact that the pH of the yeast extract greatly influences the adsorption on fuller's earth (Kringstad and Lunde, '39). At pH 5-6 we were able to demonstrate that B_6 even after three adsorptions is not quantitatively removed from the extract. At pH 1.0 factors other than B_6 are also adsorbed. At such a strongly acid reaction and under our experimental conditions, compounds are also formed which are poisonous to the avitaminotic rats. In view of the above experiments adsorption on fuller's earth does not appear to be suited for achieving a distinct separation of vitamin B_6 from the growth-promoting factor in the filtrate of a yeast extract.

Another possibility for securing this desired separation is to precipitate the B_6 by means of phosphotungstic acid. Our experiments indicate that by this procedure one can remove B_6 quantitatively from the solution.

The amount of factor B_w in certain foodstuffs was determined. Rats were allowed to subsist on an artificial diet containing sufficient amounts of thiamin, riboflavin, nicotinic acid and B_6. When growth had ceased, evidenced by mere maintenance of body weight, a test quantity of the food in question was fed daily (fig. 1). The amount of factor B_w necessary to produce an average increase in weight of 7 gm. per week was taken as defining the rat-growth unit of this factor (table 1). Cane sugar was the carbohydrate used in the basal diet. The protein source was fish meal with a low ash content. This fish meal contains only insignificant traces of the factor B_w, and it is at the same time a good source of vitamin B_6. Peanut oil was the fat used in the diet. Cod liver oil provided vitamins A and D. These experiments showed that of the substances tested the best sources of the rat-growth factor B_w were liver and yeast. Wheat germ, corn and other cereals rated as fair. Mammalian liver proved to be the best source containing at least 8 rat units per gram. Therefore the use of liver was adopted for the further experiments designed to yield a suitable concentration of this factor.

A liver concentrate prepared by extraction of a cruder extract with phenol[3] (Laland and Klem, '37) was tested on rats using our vitamin B_w-free diet containing fish meal as the source of protein. A maximum growth was obtained when 0.1 cc. liver concentrate was given daily, corresponding to 5 gm. of the original fresh liver (fig. 2). From this experiment it is evident that the factor B_w is soluble in phenol. The growth factor W of Elvehjem et al. is also soluble in phenol (Frost and Elvehjem, '39), and it is possible that these two factors

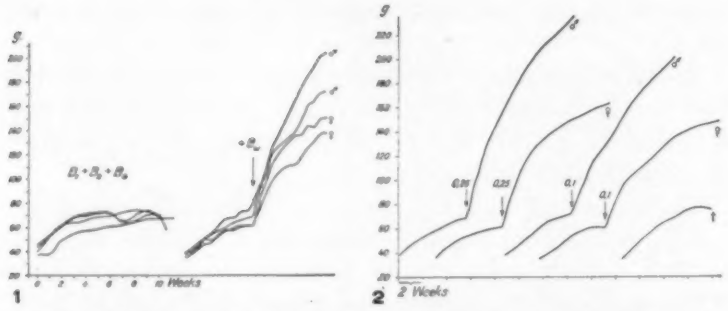

Fig. 1 Growth curves of rats subsisting on a vitamin B-complex free supplemented with B_1, B_2 and B_6 as indicated. The right-hand group of curves indicate the growth performances of rats which received in addition a supplement containing the B_w growth factor.

Fig. 2 Growth curves of rats fed the same basal diet and vitamin supplements as the animals in figure 1. When growth had decreased markedly, the rats received 0.25 or 0.1 cc., of the phenol soluble fraction of the liver extract. It is evident that this fraction contained the growth factor B_w.

are identical. In a new experiment phosphotungstic acid was added to the phenol-soluble liver concentrate. The resulting precipitate was filtered off and the excess of phosphotungstic acid removed by barium hydroxide. The barium precipitate was removed by centrifugation, and the excess of barium precipitated by means of sulfuric acid. The barium sulfate was again removed by centrifuging, and the clear solution evap-

[3] "Pernami forte" prepared by Nyegaard and Company, Oslo.

orated in vacuum to the original concentration. When rats were given 0.25 cc. of this solution, they exhibited a growth corresponding almost to that obtained when the original liver concentrate was used. This experiment shows that the factor B_w is not precipitated by phosphotungstic acid.

The growth factor B_w is almost quantitatively dissolved in an acetone-water solution. This is shown by several repetitions of the following experiment with the same result. A phenol soluble liver extract was evaporated in vacuum until of a syrupy consistency, and this syrup repeatedly extracted

TABLE 1

Distribution of the rat growth factor B_w in certain foods

FOOD	UNITS PER GRAM	FOOD	UNITS PER GRAM
Fish products		Mammalian tissue	
Fish liver meal	2.8	Liver: ox	6.0
Cod roe, canned	2.0	Liver: pork	4.0
Cod liver	1.4	Pork	0.8
Sardines, brisling, canned	0.9	Beef	0.8
Sardines, herring, canned	0.9	Miscellaneous	
Herrings, kippered, canned	0.7	Yeast: brewery	6.0
Plaice, flesh	0.6	Yeast: bakery	3.0
Cod milt	0.5	Wheat germ	2.0
Herring milt	0.5	Corn	0.7
Cod, flesh	0.2	Milk, cow's	0.15

at ordinary temperature with acetone containing 10% of water. The acetone extracts were evaporated in vacuum until syrupy in consistency and this material then dissolved in sufficient water to give the original concentration. Tested on rats fed the vitamin B_w-free diet, ¼ cc. of this solution corresponding to about 10 gm. of fresh liver gave maximum growth. The part of the liver extract not dissolved in acetone-water was also tested on rats. The rats received an amount corresponding to 10 gm. of fresh liver and showed only a very poor growth amounting to an average of about 6 gm. per week (fig. 3).

The growth factor B_w is not precipitated with mercuric chloride. In support of this statement the following experiments may be cited. The acetone soluble part was evaporated until syrupy in consistency. The syrup was dissolved in methyl alcohol and a solution of mercuric chloride in methyl alcohol added. The precipitate was removed by centrifuging, emulsified in water slightly acidified with sulfuric acid, and the mercury removed by means of hydrogen sulfide. The excess of H_2S was removed by carbon dioxide. The remaining material was then evaporated and tested on rats. It proved to have no growth-promoting properties. On the other hand, the

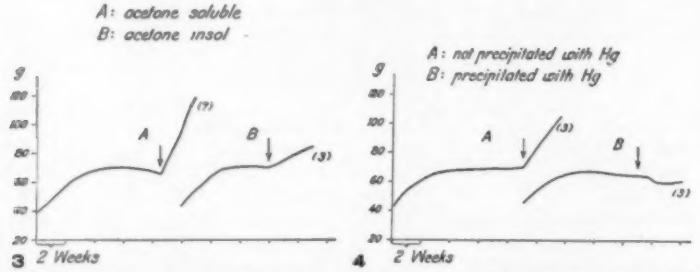

Fig. 3 Average growth curve of rats fed a B_w free diet and in addition acetone-soluble (A) or acetone-insoluble (B) part of liver extract, respectively. The figures in parentheses indicate the number of rats on the experiment.

Fig. 4 Average growth curves of rats on a B_w free diet, and receiving in addition either the filtrate after the precipitation with Hg (A) or the part of the liver extract precipitated by Hg (B). Note that the growth factor is not precipitated by treatment with Hg.

filtrate, after the mercury precipitation, was treated with H_2S to remove the mercury, and the sulfide filtered off. This filtrate was evaporated in vacuum, dissolved in water and tested on rats. Twenty-five hundredths cubic centimeters of the solution, corresponding to about 25 gm. of the original fresh liver, gave optimal growth (fig. 4).

The growth factor B_w is evidently unstable toward heat. Our original phenol soluble liver extract was heated in an autoclave for 8 hours at 120° C., and then tested on rats.

Twenty-five hundredths cubic centimeters of this autoclaved liver extract, corresponding to about 10 gm. of fresh liver, gave very poor growth. The weekly increase in body weight was on an average only 5 gm. (fig. 5).

THE ANTI GREY HAIR FACTOR B_x

When using certain diets rich in B_6 and poor in the filtrate factor (Lunde and Kringstad, '39 a), we observed a hemorrhagic condition especially on the paws, and similar to that also described by Elvehjem et al. ('37) and György et al. ('37). In addition, a striking fact was observed. In all our

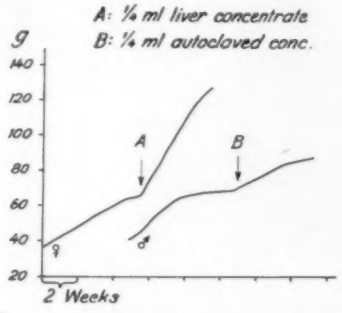

Fig. 5 Average growth curves of rats showing the adverse effect of autoclaving on vitamin B_w.

feeding tests we had been using piebald rats. We observed a peculiar greying of the black hair of these animals (fig. 6). In view of the evidence cited below, we are of the opinion that this greying of the black fur of the rats is due to lack of a new vitamin distinct from the growth-promoting factor B_w in the so-called filtrate fraction. We have provisionally designated this vitamin B_x.

In our first experiments with B_6 this syndrome was always observed when fish meal was used to cure the rat dermatitis developed by subsistence on the B_6-deficient diet. Later, several different diets were tested and it was observed that a

diet with fish meal as the source of protein gave the most pronounced greying of the hair. When using wheat germ in the diet, we encountered many cases where no greying of hair occurred, or the phenomenon was present but in a very limited degree. This was difficult to understand, in view of the fact that this greying phenomenon was first observed by Bakke et al. ('30), when using this diet. Morgan and associates

Fig. 6 Piebald rat. Note the black fur changed to grey in color. Black spots of original color are still evident.

Fig. 7 Black rat which has developed greying of the fur chiefly over the body and to a lesser extent, on the head.

('38) also reported that the greying was especially pronounced when wheat germ was used.

In all further studies black rats were used. It was noticed that the greying of the hair is always symmetrical. It is usually first observed on the head between the ears and at a certain spot on the back. In some cases it also starts on both sides of the body but always symmetrically (fig. 7). The color is not a dull grey but a more silvery grey. In some cases the

whole rat becomes silver-grey in appearance. The avitaminosis can be cured with products containing the new factor, for example, yeast and liver. Mammalian liver is especially rich in the new factor, but it is also present in fish liver.

The greying of the fur is not related to the anaemic greying of hair associated with lack of iron or copper salts. The administration to grey rats of small amounts of iron, copper and manganese salts is without effect (Lunde and Kringstad, '39 a). The new factor B_x cannot be identical with the growth factor B_w. We have observed that rats show the greying of the fur when subsisting on certain diets whether they exhibit growth or not.

The new anti-grey-hair factor B_x seems to be more heat labile than the growth factor B_w. In support of this statement, we may cite an experiment in which a yeast extract was heated for 3 hours at 100°C., concentrated in vacuum over a water bath, and then tested for both of these factors. The anti-greying substance had been lost whereas the growth factor B_w was still present in considerable amount.

Factor B_x proves to be soluble in phenol. As mammalian liver proved to be one of the best sources of the anti-grey-hair substance, our phenol soluble liver extract was tested on rats for its content of B_x. The B-free diet was supplemented with ample amounts of thiamin, riboflavin, nicotinic acid and a B_6 concentrate. After 8 to 10 weeks, the rats showed the typical greying of the black fur. The animals were then given 0.25 cc. of the liver concentrate corresponding to about 10 gm. of fresh liver. The rats immediately exhibited good growth and the fur changed to black in color within a period of 6 weeks.

The above experiment was repeated with rats allowed to subsist upon our fish-protein diet. After from 8 to 10 weeks, the rats were silvery grey in color, at which time they were given our liver concentrate as a dietary supplement. This was promptly followed by excellent growth of maximum degree. After 3 weeks the fur began to darken and after 7 weeks it had recovered its normal black color. The first change was observed on the head where the animal's grey hair was re-

placed by new black hair. As was stated previously, the greying was always symmetrical (figs. 6 and 7).

SUMMARY

From these experiments it appears evident that in addition to the known members of the vitamin B complex, the rat also needs two other factors, one a growth factor designated B_w which may be identical with the "factor W" of Elvehjem et al., the other a substance B_x required to prevent and to cure the greying of black hair.

Factor B_w is soluble in phenol but not in ether in acid solution, a fact which distinguishes it from the chick anti-dermatitis factor. B_w is easily soluble in acetone and water. It is not precipitated either by phosphotungstic acid or mercuric acetate. This factor is destroyed by autoclaving for 8 hours at 120°C. If yeast is heated for 3 hours at 100°C., a considerable amount of B_w is still present. Liver and yeast are excellent sources of this substance.

Factor B_x is likewise present in liver and yeast in large amounts. It is soluble in phenol but is not precipitated by phosphotungstic acid. This anti-grey-hair factor seems to be more heat labile than the growth factor B_w, evidenced by the fact that it is completely destroyed in yeast by heating for only 3 hours at 100°C. That the growth factor and the anti-grey-hair factor are distinct entities is further supported by the fact that they show differences in their respective distributions in the foods tested.

After this paper was read before the American Chemical Society, there appeared the communication by Oleson, Elvehjem and Hart ('39) confirming us. They conclude that this factor "is distinct from all factors of the vitamin B complex which have thus far been identified and associated with specific function in the nutrition of the rat. Further, the factor does not seem to be involved in the growth of the rat."

We also wish to add that we in new experiments have been able to show that a substance with growth-promoting properties, when tested on rats fed a B_w free diet, can be extracted

with ether from an acidified liver extract (Lunde, '39). This fact seems to indicate that we are dealing with a new growth-promoting "filtrate" factor distinct from B_w, unless the B_w-factor should be present in different forms in yeast and in liver.

We have also been able to show that the anti-grey-hair vitamin B_x is necessary for the normal development of the pelt of silver foxes (Lunde and Kringstad, '39 b).

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CYSTINE AND METHIONINE DEFICIENCY IN MOLD PROTEINS[1]

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NINE FIGURES

Little attention has been given to the nutritive value of microbial proteins, due partly, perhaps, to the difficulty of obtaining large amounts of microbial material, and partly to the fact that although many foods contain microorganisms in large numbers, still the microbial substances are small in comparison to the amounts of the food itself. This is true even in such foods as sour milk, soya sauce, sauerkraut, various kinds of cheese, etc., which contain many bacteria or molds. These organisms, dead or alive, are being consumed, however, and their food value should be known. Then, too, the ease of converting inorganic nitrogen to microbial proteins with cheap carbohydrates such as straw, sawdust, or other plant residues as energy materials, has suggested the possibility of feeding domestic animals or human beings these proteins. This was suggested, more or less fancifully, by Robertson ('20), but has actually been done by Pringsheim and Lichtenstein ('20), who fed to animals straw fortified with inorganic nitrogen fertilizer on which Aspergillus fumigatus had grown.

It is well established that many molds and bacteria can synthesize their own proteins from inorganic nitrogen. The frequently quoted work of Abderhalden and Rona ('05), who

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found that Aspergillus niger was able to synthesize straight-chain amino acids has been extended by Vorbrodt ('19, '34), Takata ('29a, b), Skinner ('34), and others, so that we know now that tyrosine, tryptophane, phenylalanine, proline, and many other amino acids are also readily formed from inorganic nitrogen by molds.

Actual feeding experiments with molds grown in inorganic nitrogen-containing media have rarely been carried out. Takata ('29a, b) grew Aspergillus oryzae in a medium with dextrin as a source of energy and $(NH_4)_2SO_4$ as the sole source of N. The mold was harvested, and fed to rats at 11, 15, and 19% protein levels in otherwise complete diets. A very slight growth on an 11% protein diet, better on a 15% diet, and a good growth on a 19% protein ration resulted. After 2 months the rats on the 19% diet no longer gained weight and were not benefited by the addition of cystine to the diet, although the protein by analysis contained only traces of cystine. The small number of animals used and the fact that the cystine was not added until late in the course of the feeding experiment left the results rather inconclusive as far as the cystine deficiency of this particular mold was concerned. The results, however, indicate that this mold may serve at least as a partial source of protein for rats. Takata considered the digestibility of the proteins to be good from the experiments which he carried out.

One of us (Skinner, '34) and Skinner (J. T.), Peterson and Steenbock ('33) independently studied the value of molds as a source of protein for animals. The few feeding experiments carried out by the former investigator were only incidental for demonstrating the synthesis of aromatic amino acids by molds. It was found that Penicillium flavo-glaucum, the mycelial growth of which was fed at a 9% protein (29% mold) level in an otherwise complete diet, supported only poor growth. A much better but still not a normally good growth was obtained at an 18% protein level, while a 9% mold protein together with either a 9% casein or a 9% Zea mays protein (20% corn-gluten meal) diet supported a normal growth

for several weeks, although each of these proteins alone is known to be deficient. It was further shown by paired-feeding experiments that cystine was the first limiting amino acid of the mold. The statistical values rather than the data were published for this last set of experiments. Skinner, Peterson and Steenbock ('33) and Gorcica, Peterson and Steenbock ('35) also showed that molds were very poor sources of proteins but could be used in conjunction with other deficient proteins to support a good growth. These authors did not find that the addition of cystine increased the value of the proteins when added to the diet either at the beginning or later in the experiment.

Several workers, from Sunderlin and Werkman ('28) on, have studied the vitamin synthesis by molds. In general it has been found that the vitamin B complex is synthesized by molds, but considerable quantities, often more than the animals willingly eat, have to be consumed to supply optimum quantities of the B complex.

There are several points on the nutritive value of mold proteins that remain to be studied. The present report will be confined to the sulfur-containing amino acid deficiencies of a few of the common molds.

EXPERIMENTAL

The molds were grown and prepared as reported previously (Skinner, '34). The medium used varied with the species[3], as

[3] Penicillium flavo-glaucum, isolated from air; Aspergillus oryzae, secured from the Department of Agricultural Bacteriology, University of Wisconsin: $Ca(NO_3)_2 \cdot 4H_2O$, 2 gm.; KH_2PO_4, 2 gm.; $MgSO_4 \cdot 7\ H_2O$, 1 gm.; $(NH_4)_2SO_4$, 2 gm.; $FeCl_3$, trace; glucose, 20 gm.; H_2O, 1000 cc.

Penicillium roqueforti, secured from the Department of Agricultural Bacteriology, University of Wisconsin; Penicillium species 1 and 2 isolated from soil, unidentified: $(NH_4)_2HPO_4$, 2 gm.; $(NH_4)_2SO_4$, 1 gm.; $MgSO_4 \cdot 7\ H_2O$, 0.5 gm.; $CaCl_2$, 0.1 gm.; KCl, 0.5 gm.; $FeCl_3$, trace; sucrose, 30 gm.; H_2O, 1000 cc.

Aspergillus nidulans, from Dr. A. T. Henrici, University of Minnesota: $(NH_4)_2HPO_4$, 2 gm.; K_2HPO_4, 1 gm.; $NH_4H_2PO_4$, 1 gm.; $(NH_4)_2SO_4$, 1 gm.; $MgSO_4 \cdot 7H_2O$, 0.5 gm.; $CaCl_2$, 0.1 gm.; $FeCl_3$, trace; sucrose, 30 gm.; H_2O, 1000 cc.

Geotrichum lactis (often known as Oidium or Oöspora lactis) isolated from sour milk: $(NH_4)_2SO_4$, 1 gm.; $(NH_4)_2HPO_4$, 2 gm.; $NH_4H_2PO_4$, 1 gm.; K_2HPO_4, 1 gm.; $MgSO_4 \cdot 7H_2O$, 0.5 gm.; $CaCl_2$ 0.1 gm.; $FeCl_3$, trace; glucose, 30 gm.; H_2O, 1000 cc.

more growth could be obtained by changing the medium slightly for the different species. In each medium, the only organic compound was either glucose, U.S.P., or cane sugar. The N source was either ammonium salts or ammonium salts and nitrates. These and the other inorganic salts used were C.P. grade. No addition was made of zinc or other salt known to be stimulating to molds in minute quantities. Obviously the traces of these salts considered necessary to molds were obtainable as impurities in the salts, the distilled water used, or the 1-liter medicine bottles used as incubation flasks, since a heavy growth was secured.

The resulting dried ground mold which had been heated for a short time at 100° C. was analyzed for total N and the protein content was estimated by use of the factor 6.25 after allowing for the nitrate and NH_3 nitrogen which remained in the mold from the medium in which the mold had grown. This factor is probably not correct as some of the mold N is undoubtedly nonprotein in nature. The rations were made with a 30% mold content in the diet, which brought the "protein" content to about 9-10%, depending upon the mold. The rest of the diet consisted of 15% fat[4], 5% salts (McCollum's 185 with iodized salts substituted for NaCl) and cornstarch. All ingredients were ground powder fine and were thoroughly mixed. Each rat received daily 2 drops of U. S. P. cod liver oil and 100 mg. of a vitamin B complex concentrate[5] in the form of pills. With some molds, after the animals had been on the diet for 30 days, 0.8 gm. dried yeast was given twice a week.

The rats were used in pairs, following the technique of Mitchell and Beadles ('30). They were litter mates of the same sex, and each pair, as closely alike as possible in weight, was put on the diet at the age of 21-28 days. One member of each pair received the ration to which was added 0.25% cystine or methionine, and the other member a similar ration with either 0.25% alanine or an equivalent amount of mold.

[4] Crisco, a vegetable fat.

[5] Harris vitamin B complex.

The amount of food each animal consumed was measured daily. The food wasted was sieved to separate the feces, and this was allowed for. Each day the animal which was willing to eat the most food was given the amount the other member of the pair had eaten the previous day, while the one which ate the lesser amount was allowed to eat ad lib. In this way the animals were truly paired, not only as to initial weight and sex, but as to food intake as well. The animals were weighed to the nearest ½ gm. every 5 days. After the first period or so, the rat with the best appetite was invariably the one which was getting the cystine or methionine diet.

If the member of each pair which was fed a sulfur-containing amino acid regularly gains more weight than the other, it may be concluded that the amino acid is beneficial. To test whether the apparent superiority of the cystine or methionine diets is real, one can either look at the charts and estimate whether the results are significant, an all too common method which is sufficient only if the data are outstanding, or one can subject the results to statistical analysis and thus ascertain objectively the chances that the results might be fortuitous. Of the three statistical methods suggested by Mitchell and Beadles ('30), only one was used.

Given the number of weighings of paired animals for each particular mold (n) and the number of times the cystine or methionine fed animal gained more than its control (a), one can calculate the index suggested by Mitchell and Beadles:

$$\frac{a - \frac{n}{2}}{\sqrt{\frac{1}{2} \times \frac{1}{2} \times n}}$$

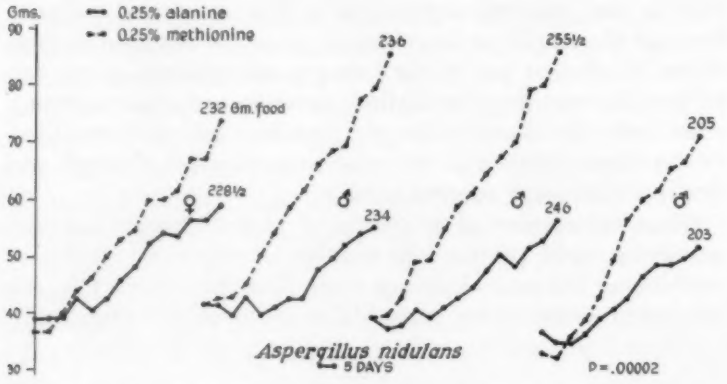
From this, using the tables of Pearson ('31), or other tables derived from them, one can find the probability (P) that chance rather than the diet caused the one member of the pair to gain more often than the other. The above formula (the number of times that the standard deviation of the frequency distribution is exceeded by the divergence between 1/2 of the number of feedings and the number of times that the cystine

or methionine-fed animal gained more than the control) may be simplified to:

$$\frac{2a - n}{\sqrt{n}}$$

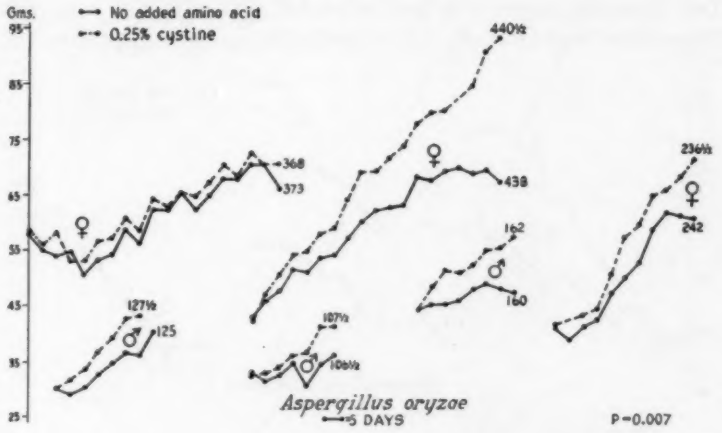
It will be noted that all our results show P to be less than 0.05. It is usually considered that any value of $P = 0.05$ or less indicates that the results are significant, and any value of $P = 0.01$ or less is considered highly significant. Here the P value may be interpreted as the probability that chance rather than differences in diets caused the cystine or methionine fed animals to gain in weight more often than the controls.

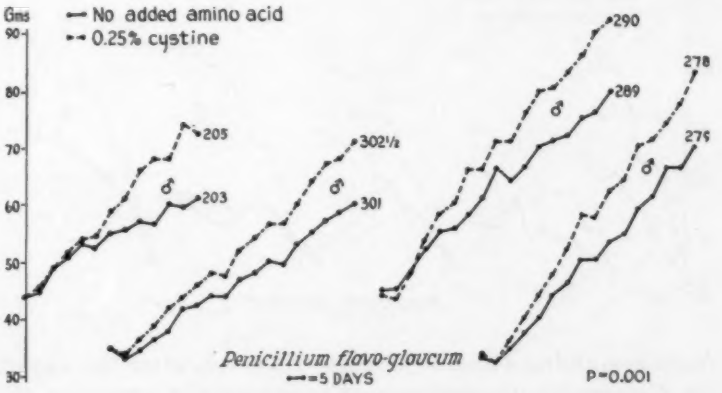
The graphs show the growth curves, the amount of food consumed, and the values of P. In all cases, it will be seen that the cystine or methionine fed animals gained significantly



more than the others except in the case of one of the unidentified Penicillium species. Here no growth resulted without cystine. These animals consumed progressively less and less food, and therefore after they had decreased in weight until there was evident danger of death, the cystine ration was given to all animals ad lib. A slight gain resulted in most cases. Here the evidence is less clear-cut that the mold was deficient in sulfur-containing amino acids, although it indicates that this may well be true. Methionine was used in place of cystine

with only some of the molds, since the work was in progress before the appearance of Rose's ('37) paper, wherein it is shown that methionine rather than cystine is essential, but

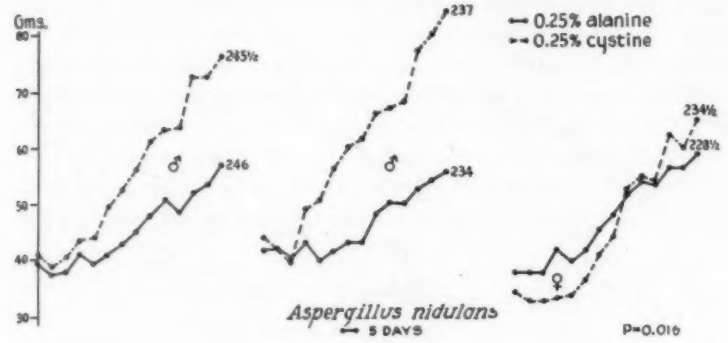


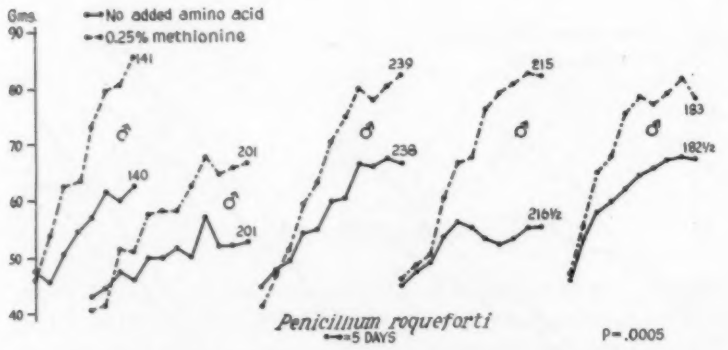


that cystine can replace methionine after the minimal requirements of this amino acid are met. The evident response to cystine showed that the molds were not entirely devoid of methionine, but were deficient in sulfur-containing amino

acids. From our results, however, it is quite possible that methionine would have demonstrated greater response than cystine did.

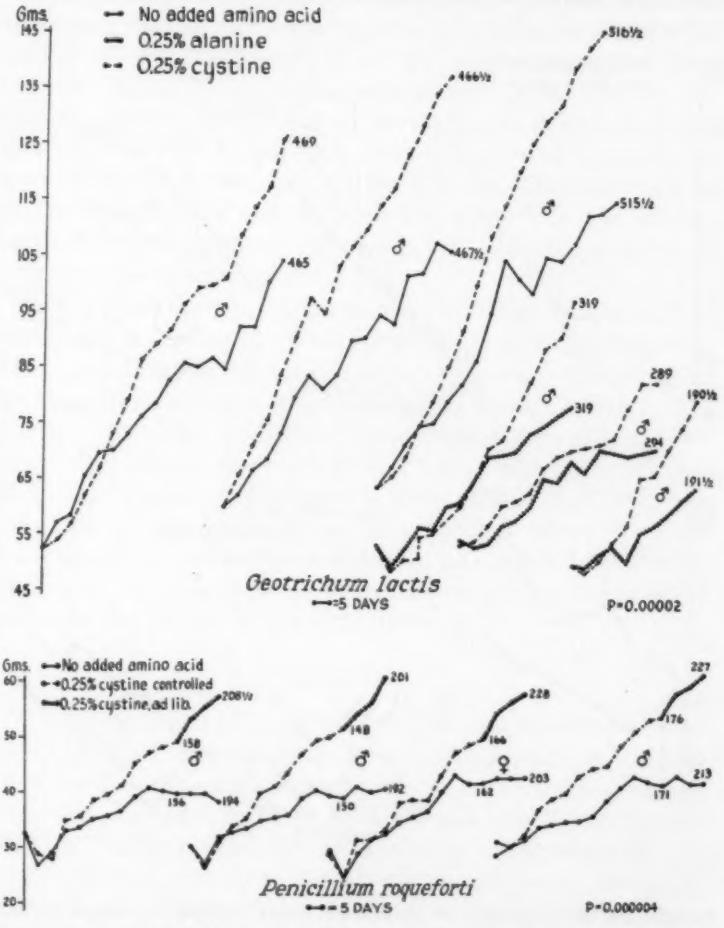
The slow growth of animals is probably partly due to the fact that the molds are not palatable. Results not reported here show that animals differ markedly in their appetites for





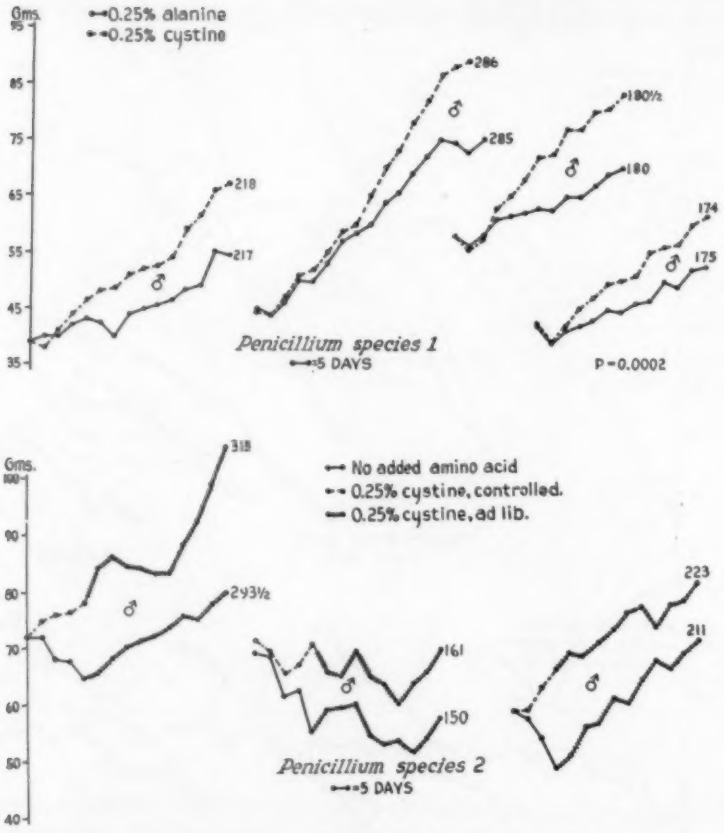
foods containing about 30% mold. However, after the apparent distaste for the mold was at least partially overcome, the animal grew fairly well on a complete diet containing mold material. One female rat fed after 28 days of age on a ration consisting of casein 15 gm., salts 5 gm., Penicillium roqueforti 30 gm. (9% protein), fat 15 gm., starch 32 gm., agar-

agar 3 gm., with the usual daily dose of vitamins, grew slowly at first, but at the age of 148 days weighed 175 gm. She was then placed in a cage with a male and became pregnant. Two



days before her litter of eight was born, when she was 173 days old, she weighed 214 gm. Other rats fed on the same diet with the same mold (which the graphs show to be one of the

poorest) did not do so well, but in every case the growth was correlated with the amount of food consumed. It is obvious that the mold was not toxic. This phase of the work will be discussed further in a later paper. All of the molds, with the



exception of Aspergillus nidulans, were tasted by the authors and found to be extremely bitter. Geotrichum lactis, which was most readily eaten by the animals, was noticeably less bitter than the others.

It should be noted that after the first week or two the greater gain in weight of the cystine or methionine fed animals occurred with animals partly starved and that the gain over the control mates continued after the control weighed considerably less; that is, the food containing cystine or methionine supported greater growth than the same amount of food without the sulfur-containing amino acid, even though the animals making the greater growth were already larger and needed more food to maintain their weight, and were willing to eat more than they got. Correcting for this difference in weight would merely show a greater spread in the graphs between the two members of a pair, and make the results appear even more significant.

It is evident that the molds studied were deficient in sulfur-containing amino acids. Whether this is true of all species of molds grown under all conditions is not known. The work of Gorcica, Peterson and Steenbock ('35) would indicate that it is not true. Our methods differed from theirs with respect to species, media, harvesting, and diets, especially in that yeast and vitamin B concentrate were added. But, with the exception of one of the Penicillium species, where the data are suggestive rather than definite, the molds which were studied by us were deficient in sulfur-containing amino acids under the conditions of our experiment.

SUMMARY

The molds, Aspergillus nidulans, A. oryzae, Geotrichum lactis, Penicillium flavo-glaucum, P. roqueforti, and two unidentified species of Penicillium were fed to rats as a sole source of protein, in diets low in protein. It was found that the addition of 0.25% cystine or methionine greatly enhanced the growth of rats over those having 0.25% alanine or an equivalent amount of mold added to the diet. It is concluded that the sulfur-containing amino acids are in too low concentration in mold proteins to promote good growth.

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THE CURE OF NUTRITIONAL MUSCULAR DYSTROPHY IN THE RABBIT BY ALPHA-TOCOPHEROL AND ITS EFFECT ON CREATINE METABOLISM

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FOUR FIGURES

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Nutritional muscular dystrophy was first described by Goettsch and Pappenheimer ('31) in rabbits and guinea pigs. They were unable to ascribe the disease to a lack of any of the known vitamins. Morgulis and his co-workers (Morgulis and Spencer, '36 a; Morgulis et al., '38) reported that at least two factors, both contained in whole wheat germ, were required to cure the disease in rabbits: a water-soluble factor present in defatted germ, and a fat-soluble factor present in the unsaponifiable matter of the oil.[1] This suggested that the fat-soluble factor might be vitamin E. Support for the hypothesis was furnished by Olcott's ('38) observation that muscle lesions similar to those in dystrophic guinea pigs occur in the paralyzed suckling young of vitamin E-deficient female rats. Barrie ('38) and Goettsch and Ritzmann ('39) have recently shown that this disorder in suckling rats is prevented by alpha-tocopherol.

On the other hand, considerable evidence existed against the postulated identity of vitamin E and the fat-soluble anti-dystrophy factor required by herbivora. The dystrophy-producing diet of Goettsch and Pappenheimer ('31) apparently

[1]Only temporary cures were obtained with these concentrates.

contains a significant amount of vitamin E, since both these workers and Morgulis et al. ('38) found that female rats grown and maintained on it exhibited "initial fertility" in the first and second generations, a phenomenon entirely inconsistent with the complete absence of the vitamin. The explanation that the rabbit requires more E than the rat seemed to be eliminated by the observation of Thomas et al. ('38), quoted in greater detail by Mattill ('38 a), that twelve rabbits on a diet deficient in E bore 137 young in 2 years. Such a performance excludes the possibility of severe dystrophy in these animals.

In addition, the muscle lesions in the suckling and adult paralyzed E-deficient rat are quite possibly secondary to degenerative changes in the central nervous system (Lipshutz, '36; Einarson and Ringsted, '38), while in the herbivora no changes in the nervous system have been detected (Goettsch and Pappenheimer, '31; Rogers et al., '31; Chor and Dolkart, '39). Further complications arose as the result of Mattill's ('38 b) report that the addition of wheat germ oil to a synthetic ration protected rabbits from dystrophy, but that the oil could not be completely replaced by potent vitamin E concentrates.

This problem was clarified by our observation, presented in a preliminary note (Mackenzie and McCollum, '39), that rabbits rendered dystrophic on diet 13 of Goettsch and Pappenheimer ('31), supplemented with defatted wheat germ, are cured by alpha-tocopherol. At that time we referred to the sharp drop in urinary creatine effected by the vitamin. Since then, Shimotori et al. ('39) have announced that alpha-tocopherol prevents muscular dystrophy in guinea pigs reared on a synthetic diet, and Morris ('39) has confirmed our observation that alpha-tocopherol cures dystrophy in rabbits.

The present paper is a detailed report of our curative experiments, together with simple criteria for diagnosing and following the development of the disease, and for detecting within a few days a response to potent supplements. Chief among these criteria is the increased creatine output

noted by Morgulis and Spencer ('36 b) in dystrophic rabbits. Data on both dystrophic and cured animals showing the relation of creatine excretion to creatinine excretion, weight changes, and food consumption are presented in figures 1, 2, 3 and 4.

METHODS

By modifying the dystrophy-producing diet 13 of Goettsch and Pappenheimer [2] through the addition of 10% defatted wheat germ, a source, according to Morgulis et al. ('38) of the water-soluble factor, we have limited our attention to the identity of the fat-soluble essential. Rabbits develop dystrophy just as rapidly on this diet as on unmodified diet 13. The defatted germ was prepared by extracting fresh wheat germ in a continuous extraction apparatus for 24 hours with cold U.S.P. ethyl ether.

Diet 13 was prepared twice a week and kept in a refrigerator. The ether-extracted wheat germ was incorporated at the time of feeding. Fresh diet was supplied the animals every 2 or 3 days.

Each rabbit was housed in an individual all-metal metabolism cage. The weight and food intake were recorded daily, and the rabbit closely examined for signs of dystrophy. Urinary creatine and creatinine were determined by the methods of Folin ('14).

[2] Diet 13.

Rolled oats (Quaker)	355 parts
Wheat bran (Pillsbury)	180 parts
Casein (Merck technical)	75 parts
Lard	80 parts
Cod liver oil (Mead Johnson & Co.)	10 parts
NaCl	10 parts
$CaCO_3$	15 parts

Ten grams of ferric chloride, U.S.P. lump, was taken up in about 125 cc. of ether and a little water, and the solution poured over the above ingredients. The mass was shaken in a closed container and allowed to stand for about half an hour. The contents were emptied upon a tray and the ether allowed to evaporate overnight. Finally there was added:

Skimmed milk powder (Merrell-Soule) 275 parts.

All supplements were fed by mouth from a 1 cc. tuberculin syringe equipped with a long blunt needle. Food was withheld for at least 2 hours before and 3 hours after supplementing to prevent mixing of the supplement and the experimental diet in the stomach. Particular importance was attached to this precaution in view of the finding of Lease et al. ('38) that vitamin A is destroyed when it is mixed with the peroxides of rancid fats in the stomach; and the finding of Weber et al. ('39) that vitamin E is not destroyed when administered separately from rancid fat so as to prevent mixing in the gastrointestinal tract. It was thought that such peroxides might be present in our experimental diet.

Most of the animals were either killed when moribund, or found within an hour after death. Autopsies were performed on these animals, and blocks routinely taken from the muscles of both thighs for microscopic examination.

EXPERIMENTAL

Since we have been unable to find a description in the literature of the behavior of dystrophic rabbits, our observations will be presented with the other diagnostic criteria employed, namely, growth, food consumption and creatine excretion.

The deficiency state. Eighteen male and female rabbits, averaging 385 gm. (270 to 470) in weight, gained on the average 220 gm. per week during the first 2 weeks on the experimental diet. The rate of gain declined during the third week, and by the fourth week had fallen to about 100 gm. The maximum weight was attained in 31 days (18 to 42), and represented an average total gain of 740 gm. During the period of gain, food consumption averaged 50 gm. per day. The disease itself has been divided into three stages.

Stage I. The initial stage of dystrophy was characterized by a rise in the daily creatine output from a normal level of less than 10 mg. to over 20 mg. This rise occurred from 18 days before to 1 day after the attainment of maximum weight. The average was 4 days prior to this time. Creatinine excretion did not increase. Following the maximum weight

there was an immediate but gradual decline in thirteen animals, and in the remainder a period of stationary weight of 5 to 12 days' duration. In either case the urinary creatine continued to rise. When the animals ceased gaining, food intake declined abruptly to an average of 33 gm. per day. The average duration of this stage, from the time of maximum weight, was 7 days.

Stage II. This stage was marked by the appearance of the first physical symptoms. The front feet were placed in the food or water jars, the front legs held stiff, and the head slightly retracted. This position was maintained for hours at a time. Several animals did not use the jars when assuming this position, but placed the fore feet well under the body between the hind legs. The second change in behavior (the first change in some cases where the above symptoms were not noted) was the ease with which the rabbits could be laid on their sides, and their slowness in righting.

By the beginning of this stage the average creatine excretion had risen to 83 mg. (50 to 150) per day. The entire group had lost an average of 100 gm. though five animals still maintained a constant weight. Food intake dropped from an average of 33 gm. to 10 gm. per day and usually reached zero either at the end of this period or early in the next period. The rate of decline in weight was accelerated. During this stage, which lasted 3.5 days (2 to 6), râles became distinctly audible in a majority of the animals.

Stage III. This, the stage of acute dystrophy, lasted 1 to 4 days and terminated in death. The animals were now readily pushed off of their feet, and regained an upright position only after a violent struggle. Some animals died while exhibiting these symptoms, while others were completely prostrated for several days before death. Such animals when picked up seemed to be devoid of all body tonus.

From the beginning of this period until it ended in death, the high creatinuria was maintained, but usually not greatly increased. The daily loss in weight ranged from 50 to 120 gm. The total loss at death averaged 250 gm. Throughout the

course of the disease there was no significant change in the creatinine output.

The most accurate indication of the onset of dystrophy is the increase in creatine excretion. While cessation of growth frequently occurs at about the same time, it can only be distinguished in retrospect from a temporary setback. However, in following the development of the disease, in estimating its severity, and in gauging the proximity of death, all of the described changes are valuable; and they are much more valuable collectively than individually. Nevertheless, special importance is attached to food intake. When an animal's daily food intake has been 10 gm. or less for 4 or 5 days, its condition is critical, and a cure is difficult if not impossible; as will be shown later, however, this is a symptom and not a cause of death.

Autopsies revealed respiratory infections varying in extent from patchy areas on one or two lobes to complete consolidation of most of both lungs. The severity of infection was not correlated with the duration or severity of the symptoms of dystrophy. The one animal free of lung infection was prostrate 2 days before death. With the exception of atrophy of the skeletal muscles, which was frequently apparent, no other macroscopic changes were observed. Microscopic examination of the thigh, diaphragm and masseter muscles revealed necrosis of the muscle fibers as described by Goettsch and Pappenheimer ('31).

The curative factor. Supplements were fed to rabbits with late stage II or stage III dystrophy. In no case was the food consumption allowed to fall below 10 gm. daily for more than 4 days. The creatine excretion exceeded 50 mg. per day. Supplements yielding negative results were refed to animals with early stage I dystrophy. If negative results were again obtained, the ability of the test animal to respond was checked by administering a supplement known to be active. Although a positive response could be detected in 2 or 3 days, the animals were continued for many weeks.

Wheat germ oil. The oil was prepared by extracting fresh wheat germ in a continuous extraction apparatus for 24 hours with cold C.P. anhydrous ethyl ether. Most of the solvent was distilled off at the water pump. The remainder was removed by warming the oil with constant stirring in an open dish on the steam bath.

Levels of 0.5 and 1 cc. per day of this oil were effective in curing dystrophy.

Ferric chloride treated wheat germ oil. Fifty grams of the above oil were dissolved in 250 cc. of ether, shaken with 7 gm. of powdered ferric chloride, and allowed to stand at room temperature overnight. The mixture was then washed repeatedly with water, the ethereal solution dried with sodium sulphate, and the ether removed at the water pump. The resulting dark reddish-brown oil possessed a decidedly acrid odor.

This oil when fed at 3 cc. per day for 7 days gave negative results.

Fractions of the unsaponifiable matter of wheat germ oil. The unsaponifiable matter from 200 gm. of wheat germ oil was partitioned into three fractions by a method previously described by us (Mackenzie et al., '38). These fractions consisted of 6.5 gm. of crystalline "sterols" insoluble in methanol at 8°C., 0.8 gm. of an orange wax insoluble in methanol packed in dry ice, and 1.5 gm. of a red-orange oil soluble in methanol packed in dry ice. The latter fraction, in which the vitamin E of the original oil was concentrated, was potent by rat assay in a single 3 mg. dose. Alpha-tocopherol itself is potent at 2 to 3 mg. The wax fraction was subjected to the original procedure and freed from a small amount of the vitamin E concentrate and 0.2 gm. of "sterols." The "sterol" fraction was recrystallized from methanol. All fractions were administered in ethyl laurate solutions.

The "sterol" and wax fractions were inactive when fed at 32 and 14 mg. per day respectively over a period of 9 days.

The vitamin E concentrate (the oil fraction) was fed at 5 and 25 mg. levels daily to rabbits with stage II dystrophy.

A positive response was detected in 2 days. Cured animals continued on these dosages for 6 to 7 weeks remained in good health and gained from 0.6 to 1 kg. Respiratory infections were absent at autopsy, and microscopic examination of striated muscles revealed no abnormalities.

Ferric chloride treated vitamin E concentrate. The E concentrate was treated in the manner described above for the wheat germ oil. Half a gram of ferric chloride was used for each 100 mg. of the vitamin concentrate. The preparation was inactive when fed at 30 mg. per day for 7 days.

From the foregoing it is apparent that the anti-dystrophy activity in the unsaponifiable matter of wheat germ oil was

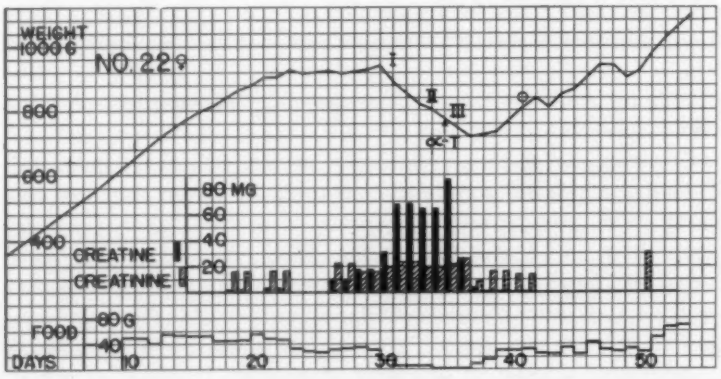

Fig. 1 Rabbit no. 22. Typical cure of dystrophy with alpha-tocopherol. The history of this animal exemplifies those cases in which the disease developed very rapidly (Roman numerals = stages of dystrophy), and in which the rise in urinary creatine was paralleled by a drastic decline in weight and food consumption. By the thirty-fourth day the animal had developed severe râles, and had difficulty in righting when placed on its side. At this time the administration of 5 mg. of alpha-tocopherol daily was commenced (↑). There was a sharp drop in creatine excretion prior to the gain in weight and food consumption. By the seventh day of treatment the animal's movements were normal (O) and the râles had disappeared. For 2 weeks food consumption and growth were below the average for a responding animal, but after that time they reached the average level.

The experiment was continued for 28 weeks, at which time microscopic examination of the biceps femoris revealed only normal muscle fibers.

confined to the fraction which, on the basis of rat assay, consisted of at least 50% vitamin E. Since a positive response could be detected in 3 days (frequently in 2 days) the effective dose of the E concentrate may be calculated as 15 mg. or less, while the ferric chloride treated concentrate was inactive at 210 mg. On the basis of this assumption, ferric chloride destroyed at least 93% of the anti-dystrophy activity.

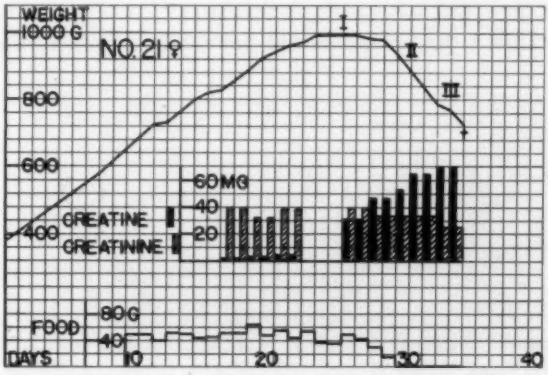

Fig. 2 Rabbit no. 21. Untreated dystrophic animal. This animal was the control of no. 22, figure 1, and like its partner developed acute dystrophy rapidly (Roman numerals = stages of dystrophy). It died (+) with stage III dystrophy on the day alpha-tocopherol therapy was begun on no. 22.

The entire apex of the left lung and part of the apex of the right lung were infected. The biceps femoris showed hyalinized areas in one-third to one-half of the muscle fibers. Many of the hyalin masses appeared to be calcified, while others were undergoing calcification or being absorbed. There was little fibrous tissue and few masses of muscle nuclei. Few polymorphonuclear leucocytes or macrophages were noted.

Similarly, wheat germ oil subjected to the same treatment lost at least 93% of its activity. While these results tend to limit the anti-dystrophy activity to a fraction of the E concentrate sensitive to ferric chloride, the possibility that the neutral fat fraction possesses some activity has not been eliminated.

Alpha-tocopherol.[3] A solution of alpha-tocopherol in ethyl laurate, 20 mg. per cubic centimeter, was employed. Fresh solutions were prepared every 2 weeks and kept in glass-stoppered bottles in the refrigerator. Both natural and synthetic preparations were used. No attempt was made to discern a difference in activity and no difference was observed.

Rabbits with stages II or III dystrophy, weighing approximately 1 kg., were cured by alpha-tocopherol at levels of 3 and 5 mg. per day. Two cured females were maintained in

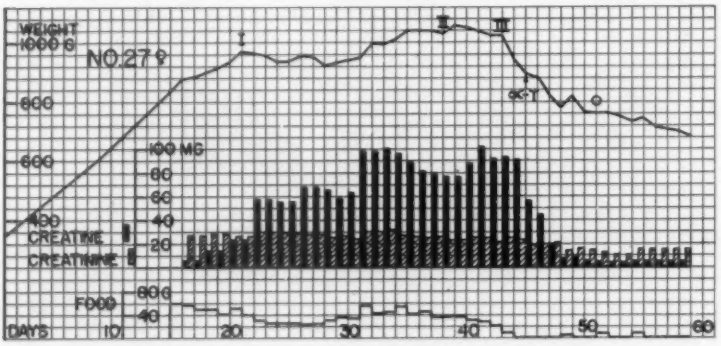

Fig. 3 Rabbit no. 27. Cure of dystrophy with alpha-tocopherol despite continued loss of weight. The history of dystrophy in this animal exemplifies those cases in which the disease developed slowly (Roman numerals = stages of dystrophy) and in which there was a long period of creatinuria during slow growth and fair food consumption. A total of 1.85 gm. of creatine was excreted over a 25-day period.

On the forty-fourth day the animal had acute dystrophy, and when pushed over could only regain its feet after a violent struggle. No râles were observed. At this time treatment with alpha-tocopherol was initiated (↑). Ten milligrams were given on the first 2 days and 5 mg. daily thereafter. The creatine output declined rapidly, but the rabbit refused food and continued to lose weight. A definite improvement in behavior was observed on the fourth day of treatment, and on the sixth day its movements were normal (O).

On the sixtieth day of the experiment (not shown in the figure) a small amount of yeast and an acetone extract of defatted wheat germ were administered and resulted in the immediate resumption of growth and food consumption.

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[3]We are indebted to Merck & Company, Inc., for the supply of alpha-tocopherol.

excellent health on the 3 mg. level for 28 weeks. At the end of that time the animals weighed 2.2 and 3.8 kg. No signs of respiratory infection were found at autopsy, and microscopic examination revealed only normal striated muscle fibers.

In addition to experiments where permanent cures were effected, the duration of a given dose was studied. Animals

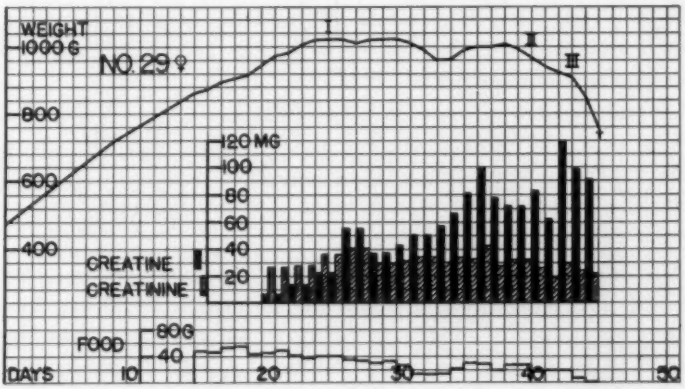

Fig. 4 Rabbit no. 29. Untreated dystrophic animal. This animal was the control of no. 27, figure 3, and like the latter exhibited a prolonged case of the disease (Roman numerals = stages of dystrophy). The animal was prostrate the day before death. Slight râles were noted at the time of death (+), the day on which therapy was started on no. 27.

The respiratory infection was relatively mild, being confined to the apex of one lung. The skeletal muscles were extremely atrophied. Sections of the biceps femoris showed large areas of fibrous tissue and masses of muscle nuclei interspersed with atrophic muscle fibers. Many of the muscle fibers surrounding these areas were hyalinized. There was no indication of calcification as in animal no. 21. A few polymorphonuclear leucocytes and macrophages were present. Similar changes were found in the diaphragm where columns of muscle nuclei were especially prominent.

weighing from 0.7 to 1.2 kg. were cured of stage III dystrophy with 3 mg. of alpha-tocopherol daily for 5 days. The daily creatine excretion fell rapidly to 10 mg. or less. In from 15 to 20 days after the first dose the daily creatine excretion had again risen to 20 mg.

If the rabbit possesses the rat's ability to store vitamin E, the daily requirement of a rabbit on the regimen employed may be put at 0.7 to 1.0 mg. per kilogram of body weight. Additional evidence that the requirement (at least for females) does not exceed this figure was obtained in a prophylactic experiment on two females. These animals, weighing 0.3 and 0.4 kg., were fed 6 mg. of alpha-tocopherol per week for 16 weeks. At the end of this time they were in good health and weighed 2.2 and 2 kg. respectively. Creatine excretion was not studied.

In some cases of very severe dystrophy the feeding of 10 mg. doses of alpha-tocopherol for 2 days, and then dropping to the permanent level, seemed to result in a more dramatic response. However, such a procedure was not essential for curing the disease.

The cure and creatine metabolism. The initial response to vitamin E therapy was a drop in the creatine output from an average level of 80 mg. per day to 46 mg. This occurred within 24 to 48 hours of the feeding of the first supplement. In the latter cases the output for the first 24 hours remained constant or increased. Following this abrupt decline excretion fell to 10 mg. or less in from 1 to 3 days. There was no significant change in creatinine excretion, except in a few cases where there was a relatively small drop of 1 or 2 days' duration, corresponding to the drop in creatine.

One or 2 days after the first decline in the creatine output the animals ceased losing weight, and began to gain from 20 to 100 gm. a day. At the same time the food intake increased, though for a day or 2 it frequently lagged behind the gain in weight by as much as 40 gm.

Definite improvement in the behavior of the animals was noted in from 2 to 4 days after commencing therapy, and in 5 to 7 days their movements were essentially normal. Cured animals resumed a normal rate of growth in 1 to 2 weeks, and gained 150 to 250 gm. a week for 7 to 10 weeks thereafter.

Individual cases selected to illustrate the response to alpha-tocopherol, and the relation of creatine excretion to creatinine excretion, food intake and growth are shown in figures 1, 2, 3 and 4. The selection of female animals for this purpose was fortuitous. Male rabbits behave in a similar manner.

DISCUSSION

The experiments described in this paper show that the muscular dystrophy produced in rabbits reared on an otherwise adequate diet can be permanently cured or prevented by alpha-tocopherol. The requirement of female rabbits for this vitamin, on the diet employed, is not greater than 1.0 mg. per kilo per day. Limited observations on male rabbits indicate a similar requirement. This approximates the fertility requirement of the female rat, if 3 mg. is accepted as the minimum dose for a 200-gm. animal. However, confirmation of the observation of Morgulis et al. ('38) that the lack of a water-soluble factor also produces dystrophy would probably lead to the establishment of a quantitative relationship between the requirements for this factor and alpha-tocopherol. In addition, definite establishment of the requirement depends on the use of a synthetic diet free of rancid fats as well as all traces of vitamin E. Even so, 1 mg. of alpha-tocopherol per kilo per day should prove adequate.

Animals cured of dystrophy that continued to receive alpha-tocopherol showed no return of symptoms even at the end of 28 weeks. The failure of Morgulis et al. ('38) and Morgulis ('38) to effect permanent cures was most likely due to their habit of incorporating the supplements in the diet. A ferric-chloride-treated diet, containing lard and cod liver oil, probably inactivates a considerable portion of any added vitamin E. The inconclusive results obtained by Mattill ('38 b) with vitamin E concentrates may have been due to an involvement of the water-soluble factor.

The experiment of B. H. Thomas et al. ('38, quoted by Mattill, '38 a), indicating the dispensability of vitamin E for reproduction in the rabbit, is difficult to explain. If their diet

was truly E-deficient, then either vitamin E is not the only fat-soluble factor that can prevent dystrophy, or very little is required if a sufficient amount of water-soluble factor is present. Otherwise their animals would all have succumbed of dystrophy long before experiments on reproduction were possible.

As a result of following changes in weight, food consumption, creatine excretion and the alacrity with which the animal moves, we have been able to detect the onset of the deficiency, follow its development and predict with considerable accuracy the time of death. The variations existing between individuals are such that the use of any one of these criteria alone is not satisfactory. Taken alone, a loss of weight would be very misleading in some cases, for several of our animals have lost 100 gm. in 1 week only to gain over 200 gm. during the next 2 weeks. While the creatine output is the most accurate index of the inception of dystrophy (and probably the extent of the lesions) it alone gives no indication of the nearness of death. For this purpose food consumption records and physical symptoms are valuable. Finally, if we were to depend solely on locomotor disturbances we would be led to conclude as was K. Thomas ('38) in a review that, "the dystrophic state sets in very suddenly after a long and uneventful prodromal period."

It is apparent that the absence of vitamin E causes a reduction in food consumption that is responsible for most of the weight loss in dystrophy. Loss of weight, however, does not invariably accompany the development of even severe dystrophy, and hence cannot be considered a cause of the disease.

The enormous increase in creatine excretion does not depend on starvation or loss in weight. This fact is particularly emphasized by those animals that develop a high creatinuria while maintaining a constant weight, and by the sequence of events following vitamin E therapy in which the creatine drops before food consumption is resumed and loss of weight ceases. Neither is the creatinuria due to a disturbance in creatinine elimination, for urinary concentration of the latter

compound remained relatively constant for each animal while that for creatine ranged from 0 to 150 mg. It may be concluded, therefore, that the creatinuria is a result of some direct effect of the vitamin deficiency on the organism. In view of this and Goettsch and Brown's ('32) observation that the creatine content of striated muscle is greatly reduced in dystrophic rabbits, it is extremely probable that the urinary creatine arises from muscle creatine.

While it seems probable that this loss of muscle creatine is a consequence of the muscle degeneration, we have no evidence that a disturbance in creatine or phosphocreatine metabolism is not the primary change in the muscle. In either case the response to vitamin E given by mouth as gauged by the drop in creatine excretion is extremely rapid, 1 or 2 days, suggesting that the vitamin acts directly on the muscle. In this connection it is interesting to recall the early observation of Evans ('32) that muscle is one of the richest animal sources of vitamin E. Verzár's ('39) recent observation that adult female rats developing paralysis and muscular dystrophy on a vitamin E-deficient diet exhibit a creatinuria which requires 200 mg. of alpha-tocopherol daily for its complete suppression is difficult to explain at the present time.

The rabbit offers excellent possibilities as an animal for vitamin E assay provided that a constant ratio exists between anti-sterility and anti-dystrophy activity. That this may not be the case is indicated by the work of Goettsch and Ritzmann ('39). Such a situation would necessitate specifying whether anti-sterility or anti-dystrophy activity is referred to when using the term "vitamin E potency." Dystrophy develops more rapidly in young rabbits than sterility in female rats, and by using creatine excretion as the basis for a test the response to a positive supplement can be detected in several days. While it may prove difficult to place this procedure on a strictly quantitative basis, a very rapid semi-quantitative method is certainly feasible.

Until very recently no species were known with certainty to require vitamin E other than the rat and mouse, and in

these two species its absence did not appear to exert a very profound effect on the well-being of the adult. Now it is known to be essential for life in the rabbit (Mackenzie and McCollum, '39), guinea pig (Shimotori et al., '39) and chicken (Dam et al., '38); and, in view of these developments, probably for life in the duck (Pappenheimer and Goettsch, '34) and goat (Madsen et al., '33). It is indispensable for the prevention of paralysis and muscular dystrophy in the suckling (Barrie, '38; Goettsch and Ritzmann, '39) and adult rat (Mackenzie et al., '39; Verzár, '39). Its elevation from a relatively obscure position among the vitamins to one of prominence would seem to warrant a reexamination of its distribution in nature.

SUMMARY

1. Nutritional muscular dystrophy in the rabbit resulting from a deficiency of a fat-soluble factor is cured by alpha-tocopherol. Continued administration of the vitamin prevents a recurrence of the disease even after acute cases. The anti-dystrophy requirement of the rabbit for alpha-tocopherol does not exceed 1.0 mg. per kilo per day.

2. The activity of the unsaponifiable matter of wheat germ oil is confined to a fraction very rich in vitamin E. The anti-dystrophy factor in this concentrate is destroyed by ferric chloride treatment.

3. Simple criteria have been established for following the development of dystrophy, and for detecting a response to potent supplements within several days.

4. A great increase in urinary creatine invariably attends the deficiency and may precede the gross symptoms by 2 weeks or more. The excretion of creatine is not due to loss of weight or starvation. A marked reduction in urinary creatine occurs within 24 or 48 hours of vitamin E administration. No comparable changes occur in creatinine metabolism.

5. Consideration of the rabbit as a test animal for the biological estimation of vitamin E is suggested.

The histological sections used in this experiment were prepared by Miss Miriam Reed.

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THE AMINO ACIDS REQUIRED FOR THE COMPLETE REPLACEMENT OF ENDOGENOUS LOSSES IN THE ADULT RAT [1]

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ONE FIGURE

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If the endogenous catabolism of nitrogen can be neither depressed nor stimulated by the feeding of amino acids or proteins, as our experimental results reported elsewhere ('40) indicate, then the changes in the output of urinary nitrogen induced by the feeding of amino acids may be given their obvious interpretation with reference to the utilization of dietary amino acids. Also, the accompanying change in the balance of nitrogen may be interpreted as a measure of the utilization of dietary nitrogen in anabolism rather than as a resultant of an indeterminate depression of endogenous losses of nitrogen associated with an incommensurable utilization of the dietary amino acids themselves. In particular, the attainment of nitrogen equilibrium by feeding an appropriate amino acid mixture to an animal may be clearly interpreted as a complete replacement of the losses of nitrogen resulting from a constant attrition of nitrogenous tissue components, not as a complete suppression of such attrition. The experiments to be reported in this paper, concerned with the determination of the amino acids required preformed in the diet for the attainment of nitrogen equilibrium, will be interpreted in this simple and direct manner.

[1] The substance of this paper was taken from a thesis submitted by E. Wise Burroughs to the Graduate School of the University of Illinois in partial fulfillment of the requirements of the degree of doctor of philosophy in animal husbandry, July, 1939.

The early investigations on this subject are reviewed in previous publications from this laboratory (Mitchell, '16; Geiling, '17). Recently Wolf and Corley ('39) have reported the results of experiments on adult rats concerned with the amino acids required for nitrogen equilibrium. They conclude that all of the amino acids except arginine that Rose ('38) has found to be essential for growth, are also essential for maintenance in the above sense, "as omission of each of these amino acids from the complete control diet has been followed by a negative balance of nitrogen, while subsequent replacement has been followed by a restoration of nitrogen equilibrium." Unfortunately there is evidence in the representative data selected for presentation that the 3-day periods into which the experiments were divided were not sufficiently long for the rats to adjust themselves to the dietary changes made. Consequently, the evidence for the indispensability of some of the amino acids, particularly lysine and histidine, is not conclusive. Also, the possibility of the conversion of a dispensable amino acid into an indispensable one at a rate adequate for the maintenance of nitrogen equilibrium if not for growth, is not even tested. Whether tyrosine can replace phenylalanine in adult nutrition, for example, cannot be answered by the experiments of Wolf and Corley.

A note by Rose and Rice ('39) concerning the significance of the amino acids in adult canine nutrition states that "the qualitative amino acid needs of the dog are identical with those of the rat" on the basis of nitrogen metabolism studies on dogs conducted apparently according to much the same plan as the experiments of Wolf and Corley, though the periods were of 7 days' duration. Until the details of these experiments become available, critical evaluation of them is obviously impossible.

There are indications, however, that the amino acid requirements of maintenance are in some particulars qualitatively different from those of growth. This evidence is very suggestive with reference to lysine. The early experiments of Osborne and Mendel ('16) showed clearly that tryptophane

was essential for both maintenance and growth, but that zein, which contains no lysine (Vickery, '38), when supplemented with tryptophane, will maintain rats at constant body weight for as long as 6 months. These experiments fall somewhat short of demonstration of the dispensability of lysine for maintenance by reason of the fact that the rations contained 28% of "protein-free milk," containing 0.7% of nitrogen (Osborne, Mendel and Ferry, '12). However, even assuming that all of this nitrogen is in the form of protein, the daily intake of protein corresponding to a food intake of 5 gm. would be no greater in amount than that contained in 200 mg. of yeast, an addendum quite commonly used in later amino acid studies in which "protein-free milk" has been dispensed with. Geiling's work ('17) also supports the view that lysine may be dispensable for maintenance.

Later experiments on the availability of lysine and its derivatives for growth have involved the feeding of zein diets to which small addenda of tryptophane and of yeast vitamin concentrate [2] have been added. In these experiments, on the basal unsupplemented diet, continued maintenance or even slow growth was observed (Berg, '36; Conrad and Berg, '37 a; Totter and Berg, '39) with rats or mice. The conclusion of Morris and Wright ('35) that there is a definite lysine requirement for the maintenance of cattle is based upon the wholly gratuitous assumption that the low biological value of wheat gluten for maintenance is the result of its low content of lysine. Osborne and Mendel ('19) have shown that the proteins of the wheat endosperm are practically as valuable for the maintenance of rats as the proteins of the whole wheat kernel, although for growth they are markedly inferior.

Many experiments on the availability of histidine and histidine derivatives for growth have shown that on the histidine-low basal diets used, containing casein hydrolysates from which the histidine was removed by mercuric sulfate or by electrodialysis, plus the usual addenda of yeast or

[2] From the Harris Laboratories, Tuckahoe, N. Y.

yeast concentrate, rats may maintain body weight for periods ranging from 20 days to 100 days (Cox and Berg, '34; Fishman and White, '36; du Vigneaud, Sifferd and Irving, '37; Conrad and Berg, '37 b). These experiments gain added significance from the fact that yeast contains only about 1% of histidine (Woolley and Peterson, '37).

Such evidence as that cited points to the possibility that lysine and histidine may not be required for the maintenance of nitrogen equilibrium in adult rats.

EXPERIMENTAL METHODS

The method used in determining the amino acids required to replace the endogenous losses of nitrogen in adult rats was similar to that used by Wolf and Corley, namely, the determination of the effect on the nitrogen balance of the successive withdrawal of individual amino acids, attention being confined to the ten amino acids considered by Rose to be indispensable for growth. However, certain important differences in procedure should be noted:

1. The complete amino-acid mixture used contained not only the ten indispensable acids, but also as many of the dispensable ones as were available. Thus, the possibility of the conversion of a dispensable acid into an indispensable one at a rate sufficiently rapid for the maintenance of nitrogen equilibrium, though not rapid enough for appreciable growth, could be explored.

2. No supplements of the B-complex vitamins were fed during the experimental periods with the exception of a daily dose of 0.1 mg. of crystalline thiamin hydrochloride. The necessity of adding other nitrogen-containing vitamin supplements was obviated by incorporating yeast concentrate [3] in the diet of the preliminary feeding period of 12 to 14 days. Advantage was thus taken of the known capacity of the animal body to store the various factors in the vitamin B_2 complex, especially in the liver. It was hoped that this storage would

[3] See footnote 2, p. 365.

not be depleted during the period of experimental feeding, but if depletion did occur there seems to be sufficient evidence in the literature (Karr, '20; Fixsen, '30; Kon, '31) to the effect that the metabolism of nitrogen would not be adversely affected provided the intake of food was sufficient to cover the energy requirements.

3. Throughout the experimental feeding periods with each rat, the same amount of food was given. Early in the investigation it was found that not only were the incomplete diets unpalatable, but the same was also true of the complete mixtures. The rats would voluntarily eat a fair portion of their food, but not in amounts sufficient to maintain body weight, nor would they eat even approximately the same amount each day. Since the consumption of an adequate and a constant amount of food is of paramount importance in a nitrogen balance study, each rat was force-fed his entire ration twice daily by a method patterned after the technic that Wolfe ('38) used in administering amino acid mixtures to rats.

Adult female albino rats, weighing approximately 200 gm. (170 to 250 gm.), served as subjects of the experiment. They were first fed a standardizing diet for a period of at least 12 to 14 days. This ration contained only about 5% of protein and was well supplied with the B vitamins. The purpose of this period was not only to replenish the stores of the B_2 vitamins, but also to deplete the stores of "deposit protein" and to reduce all rats to a uniform low level of protein nutrition.

Following the standardizing period the rats were placed upon a nitrogen-free diet containing, in per cent: starch 63, sucrose 10, salt mixture (modified Osborne and Mendel) 5, lard 10, butterfat 10, cod liver oil 1.5, and wheat germ oil 0.5. In addition, 1 or 2 gm. of sucrose were given to each rat daily, the amount depending on the ability of the rat to maintain body weight. On the second day on this diet, each rat was given a complete amino-acid mixture equivalent to 80 mg. of nitrogen daily, and the supplemented ration was

fed until daily positive and approximately uniform nitrogen balances were obtained, ordinarily requiring from 5 to 10 days. The level of amino-acid feeding was selected on the basis of preliminary experiments as being adequate for nitrogen equilibrium but not excessive. The nitrogen in the complete amino acid mixture was divided equally among the following twenty amino acids: *dl*-threonine, *dl*-isoleucine, *l*-tryptophane, *dl*-valine, *dl*-methionine, *dl*-lysine, *l*-histidine, *dl*-phenylalanine, *dl*-leucine, *d*-arginine, *l*-cystine, *l*-tyrosine, glycine, *dl*-alanine, *dl*-serine, *dl*-norleucine, *dl*-aspartic acid, *dl*-glutamic acid, *l*-proline, and *l*-hydroxyproline.

In the next experimental period amino acids were withdrawn individually from the mixture, to determine the effect on the nitrogen balance. The incomplete mixtures were also fed at a rate equivalent to 80 mg. of nitrogen per rat per day. In this manner the dispensability for nitrogen equilibrium of the ten amino acids considered indispensable for growth by Rose was tested, and also the dispensability of cystine and tyrosine. These deficient mixtures were fed until uniform daily nitrogen balances were obtained, requiring usually from 3 to 5 days, though in a few cases the period was extended to 8 days.

The rats were then returned to the complete mixture of amino acids used in period 1. This period was continued until consistent positive nitrogen balances were secured, or until it was evident that positive balances could not be obtained. Only in the former case was the test considered successful.

Throughout these three periods, each rat received the same amount of food daily, generally 8 gm., and the same amount of nitrogen, 80 to 84 mg., of which 3 to 4 mg. were supplied by the basal diet. The metabolism cages and the methods of collection and analysis of excreta are described elsewhere (Burroughs, Burroughs and Mitchell, '40). However, in the experiments to be described below, analyses were made on the daily samples of urine. The feces were composited and analyzed for the various experimental periods.

The proof of the purity of the amino acids used in these experiments rests upon the analyses for total nitrogen, or for amino nitrogen by the Van Slyke method. The agreement with the theoretical percentages was within 0.2%, with the exception of *l*-proline (0.25%) and of *d*-arginine monohydrochloride. In the latter case the discrepancy was found to be due to a contamination with mineral matter amounting to 5%. In order best to avoid contamination of one amino acid with another, synthetic racemic mixtures were used whenever obtainable.

DISCUSSION OF EXPERIMENTAL RESULTS

Representative results obtained with the twelve amino acids that were studied by the method of successive withdrawal from the complete amino-acid mixture are presented in the chart. With reference to these results, the following points of interest may be mentioned.

The withdrawal of only five amino acids produced a marked negative nitrogen balance in the experimental rats. Somewhat in the order of the magnitude of the negative balance produced, the amino acids are: threonine, isoleucine, valine, tryptophane and methionine. With the first three of these acids, the change from a positive to a negative nitrogen balance was abrupt; with tryptophane and methionine a lag of 1 to 3 days occurred after the withdrawal of the acid before the loss of nitrogen from the body exceeded the intake. Withdrawal of methionine from the ration produced a progressive increase in negative nitrogen balance throughout the period during which such balances were obtained. There is no question but that these five amino acids are essential dietary constituents for the maintenance of nitrogen equilibrium in the adult rat.

The relationship between methionine and cystine revealed by these studies is shown by the data summarized in table 1. The delayed appearance of the disturbance in nitrogen metabolism occasioned by the withdrawal of methionine from the diet is shown best in the case of rat 24, which maintained

a distinctly positive balance for 4 days on a methionine-free diet. The data on rats 24 and 51 show that a methionine deficiency cannot be compensated for by the addition of cystine. On the other hand, a negative balance of nitrogen brought about by the withdrawal of cystine from the diet (rat 36) can be corrected by an addition of methionine. In the case of rat 38, the same effect was shown, but the period

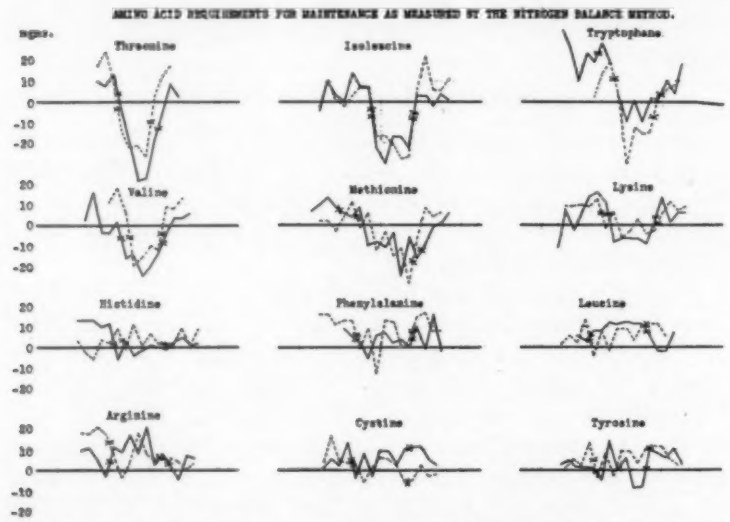

Fig. 1 Each pair of lines represents nitrogen balances with two female rats. Points between x——x represent days on respective deficient amino acid mixtures. Points preceding or following x——x represent days on complete amino acid mixture.

was not continued long enough, apparently, for equilibrium to be established. The experiments indicate clearly that there is a body requirement for both cystine and methionine, but that dietary cystine can cover only the cystine requirement, while dietary methionine can cover the requirements for cystine as well as for methionine.

The evidence for the dispensability for the maintenance of nitrogen equilibrium of some of the amino acids that are

indispensable for growth requires more complete presentation than is afforded by the chart. Hence, in tables 2 to 5, inclusive, the period averages for individual rats have been tabulated, embodying all of the evidence obtained with reference to

TABLE 1

The significance of methionine and cystine in the endogenous metabolism

RAT NO.	LENGTH OF PERIOD	AMINO ACIDS OMITTED FROM THE COMPLETE MIXTURE	INITIAL BODY WEIGHT	GAIN OR LOSS IN WEIGHT	AVERAGE VALUES PER DAY:			
					Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen balance
	days		*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
20	6	None	198	0	82.7	69.4	8.4	+4.9
	4	Methionine		−3	83.0	78.6	11.8	−7.4
	8	None		−3	82.7	72.3	7.4	+3.0
24	3	None	190	+2	81.2	62.9	8.1	+10.2
	4	Methionine		+1	82.4	66.9	8.6	+6.9
	3	Methionine		+3	82.4	83.6	8.6	−9.8
	4	Methionine[2]		+3	82.4	86.7	8.6	−12.9
	4	None		0	81.2	67.2	12.2	+1.8
51	5	None	215	+11	83.8	69.5	11.2	+3.1
	2	Methionine [1]		+1	83.4	66.4	14.5	+2.4
	5	Methionine [1]		−4	83.4	83.1	14.5	−14.2
	4	None		−4	83.8	68.7	8.9	+6.2
36	4	None	210	+3	81.2	76.1	7.2	−2.1
	4	Cystine		−10	82.3	97.1	9.2	−24.0
	1	Cystine [2]		−2	82.3	94.9	9.9	−22.5
	2	Cystine [2]		−1	82.3	65.4	9.9	+7.0
	4	None		0	81.2	72.4	8.3	+0.5
38	4	None	231	+1	81.2	68.7	11.6	+0.9
	4	Cystine		−8	82.3	96.4	12.5	−26.6
	3	Cystine [2]		−4	82.3	76.3	12.8	−6.8
	4	None		+4	81.2	69.6	9.4	+2.2

[1] In these periods the proportion of cystine in the amino acid mixture was doubled.

[2] In these periods the proportion of methionine in the amino acid mixture was doubled.

these amino acids except the few unsatisfactory experiments in which a positive nitrogen balance could not be established in the final period on the complete amino-acid mixture.

The withdrawal of lysine from the diet (table 2) was associated with only small losses of nitrogen daily, and when

the norleucine content of the ration was doubled, the average loss of nitrogen over a 7-day period was only 1.5 mg. daily. For the individual days of this period the balances ranged from −7.2 to +6.4 mg. of nitrogen. For rats 21, 22 and 50, the average daily excretion of urinary nitrogen definitely increased in the second experimental period when lysine was withdrawn from the diet, but in the case of rat 75, for which the nitrogen intake was reduced to 33.5 mg. daily, less than the endogenous loss of nitrogen for a rat of this size, the

TABLE 2

The significance of lysine in the endogenous metabolism

RAT NO.	LENGTH OF PERIOD	AMINO ACIDS OMITTED FROM COMPLETE MIXTURE	INITIAL BODY WEIGHT	GAIN OR LOSS IN WEIGHT	AVERAGE VALUES PER DAY:			
					Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen balance
	days		*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
21	6	None	196	0	82.7	66.6	10.4	+5.7
	7	Lysine		−5	82.1	75.0	11.2	−4.1
	4	None		−4	82.7	68.1	9.6	+5.0
22	4	None	193	+2	82.7	63.2	8.0	+11.5
	5	Lysine		−2	82.1	82.3	7.4	−7.6
	5	None		−2	82.7	68.2	9.2	+5.3
50	5	None	225	+4	83.8	67.3	6.8	+9.7
	7	Lysine [1]		−3	82.6	73.2	10.9	−1.5
	4	None		−2	83.8	67.2	8.6	+8.0
75	4	None	183	−4	33.5	41.3	10.2	−18.0
	5	Lysine [1]		−3	33.5	42.9	10.2	−19.6
	4	None		−2	33.5	42.6	9.3	−18.4

[1] During this period, the proportion of norleucine in the amino acid mixture was doubled.

urinary nitrogen was not appreciably increased by the withdrawal of dietary lysine. The evidence suggests rather strongly, but unfortunately does not prove, that lysine is a dispensable dietary component in the maintenance of the nitrogenous integrity of the tissues.

As regards phenylalanine and tyrosine, the data in table 3 show clearly (1) that a diet lacking either of these amino acids but containing proper amounts of the other is compatible with nitrogen equilibrium, (2) that the withdrawal of

TABLE 3

The significance of phenylalanine and tyrosine in the endogenous metabolism

RAT NO.	LENGTH OF PERIOD	AMINO ACIDS OMITTED FROM COMPLETE MIXTURE	INITIAL BODY WEIGHT	GAIN OR LOSS IN WEIGHT	AVERAGE VALUES PER DAY: Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen balance
	days		*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
30	3	None	197	+1	81.2	57.1	7.7	+16.4
	8	Phenylalanine		+1	80.4	78.0	6.9	—4.5
	3	None		—4	81.2	70.5	6.9	+3.8
25	2	None	196	—5	81.2	68.0	6.5	+6.7
	7	Phenylalanine [1]		—3	80.4	72.5	6.0	+1.9
	4	None		—3	81.2	66.9	8.3	+6.0
53	5	None	207	+3	83.3	62.8	6.8	+13.7
	7	Phenylalanine [1]		0	82.1	68.4	10.6	+3.1
	4	None		—1	83.3	63.0	8.4	+11.9
76	3	None	184	—1	33.5	39.0	11.6	—17.1
	5	Phenylalanine		—4	33.5	40.3	8.4	—15.2
	4	None		—2	33.5	44.5	12.2	—23.2
31	5	None	185	—6	81.2	71.1	8.1	+2.0
	6	Tyrosine		—3	83.1	76.0	7.9	—0.8
	5	None		—4	81.2	65.8	7.9	+7.5
32	5	None	195	0	81.2	72.4	8.7	+0.1
	6	Tyrosine		—4	83.1	76.4	9.5	—2.8
	5	None		0	81.2	72.8	9.5	—1.1
62	4	None	236	+11	83.8	68.2	10.0	+5.6
	4	Tyrosine, phenylalanine		—3	83.3	78.2	9.7	—4.6
	4	Tyrosine, phenylalanine		—5	93.2	82.4	17.7	—6.9
63	4	None	242	+6	83.8	67.9	7.0	+8.9
	4	Tyrosine, phenylalanine		0	83.3	77.6	9.4	—3.7
	4	Tyrosine, phenylalanine		+2	93.2	99.0	7.4	—13.2
	4	Tyrosine [2]		—2	94.2	70.4	11.4	+12.4
	4	Phenylalanine [1]		0	92.4	79.6	11.4	+1.4

[1] During these periods the proportion of tyrosine in the amino acid mixture was doubled.

[2] In this period the proportion of phenylalanine in the amino-acid mixture was doubled.

phenylalanine from an amino-acid mixture, fed at a low level of intake and containing a proper amount of tyrosine, does not induce an increase in the nitrogen output in the urine (rat 76), but (3) that the withdrawal of both tyrosine and phenylalanine from the diet simultaneously is incompatible with nitrogen equilibrium. The evidence quite clearly indicates that phenylalanine and tyrosine are interchangeable in adult nutrition.

TABLE 4

The significance of leucine in the endogenous metabolism

RAT NO.	LENGTH OF PERIOD	AMINO ACIDS OMITTED FROM COMPLETE MIXTURE	INITIAL BODY WEIGHT	GAIN OR LOSS IN WEIGHT	AVERAGE VALUES PER DAY:			
					Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen balance
	days		*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
23	4	None	193	+3	81.2	56.7	8.8	+15.7
	6	Leucine		−1	82.5	55.7	6.7	+20.1
	5	None		−2	81.2	61.0	5.4	+14.8
24	2	None	191	−3	81.2	68.7	8.2	+4.3
	7	Leucine		+1	82.5	64.9	6.5	+11.1
	4	None		−2	81.2	71.1	7.9	+2.2
48	4	None	210	−1	83.0	69.6	8.0	+5.4
	7	Leucine, tyrosine, cystine [1]		−2	82.1	65.4	12.1	+4.6
	4	None		−6	83.0	66.1	9.6	+7.3
49	4	None	203	+1	83.0	67.8	8.5	+6.7
	7	Leucine, tyrosine, cystine [1]		−4	82.1	72.6	9.1	+0.4
	4	None		−3	83.0	74.3	10.1	−1.4
70	3	None	182	−7	82.2	64.8	8.5	+8.9
	4	Leucine, norleucine		−9	81.9	81.1	8.5	−7.7
	3	None		+6	82.2	65.4	8.2	+8.6

[1] During these periods the proportions of both phenylalanine and methionine in the amino-acid mixture were doubled.

The feeding of a leucine-free amino-acid mixture to rats 23 and 24 (table 4) neither increased the nitrogen output in the urine nor induced a negative balance of nitrogen during periods of 6 or 7 days duration. To meet the objection that leucine may be present in some of the naturally-occurring amino acids as a contamination without modifying materially

their observed contents of nitrogen, two rats, nos. 48 and 49, were fed a diet lacking leucine and also tyrosine and cystine, the amino acids most likely to be contaminated with leucine. Again, positive nitrogen balances were obtained throughout 7-day periods. It may be concluded, therefore, that leucine is dispensable from the diet for the mere maintenance of nitrogen equilibrium. The results on rat 70, indicating a negative nitrogen balance when both leucine and norleucine are withdrawn from the diet simultaneously point toward the conclusion, indefensible from the biochemical standpoint, that norleucine may be convertible into leucine in the body, on the assumption that norleucine itself is a non-essential dietary component.

The metabolism data summarized in table 5 demonstrate that neither histidine nor arginine are essential in the maintenance of nitrogen equilibrium. In order to insure the validity of this conclusion, the histidine-free amino acid mixtures fed to rats 52 and 64, as well as all naturally-occurring amino acids in them, were examined for the presence of histidine by means of the Knoop bromine test. The test was negative in all cases. Also, the withdrawal of both histidine and arginine failed to induce a negative nitrogen balance in rat 69.

The above experiments indicate, with more or less certainty, that the adult rat needs only the following amino acids in the function of replacing completely the loss of nitrogen in the endogenous catabolism: threonine, isoleucine, tryptophane, valine, methionine, phenylalanine or tyrosine, and leucine or norleucine. The evidence with reference to the replaceability of leucine by norleucine is admittedly weak, and that for the dispensability of lysine is not particularly strong, and here also the function of norleucine as a precursor or substitute may enter in. A crucial test of this proposition would be one concerned with the adequacy of such an amino acid mixture in replacing the endogenous losses of nitrogen in adult rats. A test on three female rats was therefore undertaken with the following mixture, the percentages indicating the distribution of nitrogen: threonine 15, isoleucine 15,

tryptophane 6, valine 12, methionine 12, norleucine 30, and tyrosine 10. The proportions of the amino acids in this mixture were quite arbitrarily selected. The rats, previous to the test, had been subsisting on the basal nitrogen-free diet.

The data obtained with one of the rats, no. 71, during an 8-day feeding period, were quite satisfactory in establishing

TABLE 5

The significance of histidine and arginine in the endogenous metabolism

RAT NO.	LENGTH OF PERIOD	AMINO ACIDS OMITTED FROM COMPLETE MIXTURE	INITIAL BODY WEIGHT	GAIN OR LOSS IN WEIGHT	AVERAGE VALUES PER DAY:			
					Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen balance
	days		*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
13	3	None	218	+1	82.5	64.5	10.2	+7.8
	9	Histidine		−6	82.5	54.2	10.1	+18.2
	3	None		−3	82.5	60.7	6.5	+15.3
52	5	None	215	−1	83.3	63.6	7.7	+12.0
	6	Histidine [1]		−5	82.4	74.4	9.1	−1.1
	4	None		−2	83.3	72.0	9.0	+2.3
64	3	None	251	−2	93.7	77.1	11.6	+5.0
	6	Histidine [1]		+1	92.7	77.5	11.6	+3.6
	4	None		−6	93.7	78.2	10.1	+5.4
35	4	None	210	+1	81.2	67.4	8.6	+5.2
	6	Arginine		0	81.5	63.0	6.8	+11.7
	5	None		−2	81.2	71.3	6.6	+3.3
39	4	None	207	+1	83.0	54.2	10.1	+18.7
	7	Arginine [2]		−5	81.6	64.6	10.9	+6.1
	4	None		−1	83.0	66.8	13.2	+3.0
69	3	None	172	−1	82.2	61.0	7.7	+13.5
	5	Arginine, histidine		−3	82.6	63.5	6.8	+12.3
	3	None		0	82.2	63.1	6.3	+12.8

[1] In these periods the proportion of arginine in the amino-acid mixture was doubled.

[2] In this period the proportion of histidine in the amino-acid mixture was doubled.

the adequacy of the amino-acid mixture (table 6). Positive nitrogen balances were obtained on all but 2 of the 8 experimental days, and the average balance for the period was +2.1 mg. of nitrogen per day. The body weight was also maintained after a slight initial loss. The other two rats,

on the same amino-acid mixture (containing a new supply of four of the amino acids) lost weight rapidly (3 to 5 gm. daily), exhibited a pronounced negative nitrogen balance and seemed to be in constant agony. That these events were not the result of an amino-acid deficiency is shown by the fact that they were not alleviated by the addition of histidine,

TABLE 6

The nitrogen metabolism of rats on a simplified amino-acid mixture[1]

DAY	BODY WEIGHT	FOOD INTAKE	DAILY NITROGEN METABOLISM:			
			Intake	Urine	Feces	Balance
	gm.	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
			Rat 71			
1	175	7.0	81.3	71.5	8.2	+1.6
2	174	7.0	81.3	68.0	8.2	+5.1
3	175	7.0	81.3	71.5	8.2	+1.6
4	172	7.0	81.3	76.0	8.2	—2.9
5	172	7.0	81.3	65.9	8.2	+7.2
6	172	7.0	81.3	73.7	8.2	—0.6
7	172	7.0	81.3	71.0	8.2	+2.1
8	172	7.0	81.3	70.2	8.2	+2.9
Average	173	7.0	81.3	71.0	8.2	+2.1
			Rat 77			
1	188	7.0	80.9	51.2	6.8	+22.9
2	188	7.0	80.9	79.9	6.8	—5.8
3	189	7.0	80.9	72.4	6.8	+1.7
4	188	7.0	80.9	104.9	6.8	—30.8
5	186	7.0	80.9	70.7	6.8	+3.4
6	188	7.0	80.9	72.4	6.8	+1.7
Average	188	7.0	80.9	75.2	6.8	—1.1
			Rat 78			
1	193	7.0	80.9	67.1	5.7	+8.1
2	194	7.0	80.9	79.1	5.7	—3.9
3	194	7.0	80.9	76.3	5.7	—1.1
4	194	7.0	80.9	82.4	5.7	— 7.2
5	192	7.0	80.9	77.0	5.7	—1.8
6	194	7.0	80.9	75.5	5.7	—0.3
Average	194	7.0	80.9	76.2	5.7	—1.0

[1] The distribution of nitrogen in the amino acid mixture given to rat 71 was as follows in per cent: threonine 15, isoleucine 15, tryptophane 6, valine 12, methionine 12, norleucine 30, and tyrosine 10.

The mixture fed to rats 77 and 78 was the same as the above mixture with the exception of the substitution of leucine for norleucine.

lysine, leucine and phenylalanine to the diet. They suggest a toxicity of some constituent of the diet, and on canvassing the situation it appeared that the most likely culprit was the norleucine, since Womack and Rose ('36) had reported somewhat similar (though less severe) experiences with this amino acid (see also Rose, '38).

Therefore, the amino-acid mixture was changed by the substitution of leucine for norleucine, and the experiment was repeated on two other rats, nos. 77 and 78, with the results assembled in table 6. The irregular nitrogen balances of rat 77 on the first and the fourth day of the experiment are quite unusual and inexplicable. For both rats the average daily nitrogen balance for the 6-day period was very close to zero, i.e., —1.2 and —1.0 mg., indicating essential equilibrium, perhaps within the limits of error of the methods used. The authors believed that in all probability positive balances would have been obtained if the intake of the simplified amino-acid mixture had been raised. In any case, the positive results obtained with rat 71 are sufficiently clear-cut to establish the adequacy of the amino acids fed.

The fact that the rat needs ten amino acids in the diet for growth and only seven for the replacement of endogenous losses can be explained on the same general basis as that used by Rose (Westerman and Rose, '28) in explaining the inability of α-di-hydroxy-β-dithiodipropionic acid to replace cystine in the diet, and later (Rose, '38) in the evaluation of arginine for growth. In the former case, the negative experimental results obtained were explained as follows: "Either the amino acid cannot be formed from the corresponding hydroxy acid under the conditions of our experiments, or its synthesis is not sufficiently rapid to meet the growth requirements of the organism." In the latter case, in view of the fact that the body's ability to synthesize arginine had been established (Scull and Rose, '30), but that in the absence of arginine from the diet rats "invariably gain only about 70 to 80% as much as controls which receive this amino acid" (Rose, '38), arginine is classed as an indispensable amino

acid in the sense that it "cannot be synthesized by the animal organism, out of the materials ordinarily available (Cox and Rose, '26) at a speed commensurate with the demands for normal growth." In the same vein, he predicts: "For adult animals it may prove to be dispensable inasmuch as its synthesis may proceed at a rate equal to the requirements of maintenance alone." His own results, but recently announced (Rose and Rice, '39) confirm this prediction, as do those reported in this paper.

The hypothesis that certain of the body's synthetic capacities set the pace for growth, analogous to Robertson's ('23) "master reactions," and may even limit growth to a rate that is subnormal compared to what may be attained under adequate dietary conditions, receives support from evidence outside the field of protein metabolism. The tissues of the guinea pig apparently can synthesize ascorbic acid (Wachholder, Baucke and Podesta, '39), but certainly not at a rate to permit normal growth and health. On the other hand, the mouse, in spite of its ability to synthesize ascorbic acid (Beard, '25–'26; Harde and Wolff, '34) may not be able to grow and maintain health on diets devoid of the vitamin (Kleiner and Tauber, '36). Such evidence suggests the possibility that specific differences with respect to vitamin requirements may not be as sharp as is commonly supposed, but may be an expression merely of differential rates of synthetic reactions.

In lactation, an analogous situation exists with reference to the synthesis of fat. The ability of the body to synthesize fat from carbohydrate was one of the first synthetic reactions demonstrated in animal nutrition. However, a modicum of fat in the diet is essential for the maximum performance of this function (Maynard and McCay, '32; Mackenzie, Mackenzie and McCollum, '39).

Thus, the results of this investigation, insofar as they differ from the growth investigations of Rose, may be explained on the basis of the relation between the rate of synthesis of an amino acid and the physiological demand for it in growth

as compared to the reconstruction of tissue constituents containing it necessitated by their endogenous catabolism.

Lysine may be synthesized at a rate compatible with the replacement of endogenous losses of it, but incompatible with the demands of growth or of reproduction in the female (Pearson, '37; Lafon and Veillet, '38). The same situation may exist with reference to histidine and leucine. However, the alternative interpretation that the body does not need these amino acids in the replacement of endogenous losses is seemingly as valid as the one just given, except possibly in the case of histidine. Besides its occurrence in body proteins, histidine occurs in, or is related to, such compounds as ergothionine, carnosine and histamine that occur in the tissues and would presumably be involved in the endogenous catabolism.

The apparent interchangeability of phenylalanine and tyrosine may be at such a slow rate as to be without significance in growth, although adequate for the demands for nitrogen equilibrium. The conversion of phenylalanine into tyrosine in the body is strongly suggested by the experimental findings of Embden and Baldes ('13) and of Kotake, Masai and Mori ('22), without, however, any implication that this is necessarily the normal pathway for the metabolism of phenylalanine. However, no evidence for the conversion of tyrosine to phenylalanine has ever been obtained and the fact that the reduction of phenolic substances such as tyrosine to unsubstituted benzene derivatives has, according to Dakin ('22), not yet been observed in the animal organism, suggests "that the nuclear oxidation of phenylalanine is an irreversible reaction." The facts that phenylalanine but not tyrosine is required for growth (Womack and Rose, '34), that either one may serve for the maintenance of nitrogen equilibrium (this investigation), and that phenylalanine may be converted to tyrosine in the body while the inverse is improbable, can best be reconciled on the assumption that the body needs both amino acids for the synthesis of protein in growth, but needs only tyrosine in the replacement of endogenous

losses. The requirements of growth can be covered by phenylalanine alone and only partially by tyrosine. The tyrosine requirement for nitrogen equilibrium can be covered by either tyrosine itself or by phenylalanine, which may yield it in metabolism. This interpretation receives added support from the fact that Womack and Rose were able to secure maintenance of body weight for 20 days in four rats on a diet containing tyrosine but no phenylalanine except in the vitamin addenda used.

A logical extension of these conceptions leads to the conclusion that the amino acid requirements for different species may differ, not because of qualitative differences in their synthetic capacities, but because of differences between the rate of supply and the rate of demand. Thus, on an arginine-free diet, the synthesis of arginine in the rat proceeds at such a rate that growth can occur at 75% of its normal rate. In the chick, the restriction of growth is even more severe, since even 20% of casein in the diet apparently fails to provide sufficient arginine for maximum growth (Klose, Stokstad and Almquist, '38). It seems quite probable, that the amino acids that must be present in the diet preformed to permit maximal growth would be fewer in number for a slowly-growing species, such as the human species, than for a rapidly-growing species, such as the rat.

CONCLUSIONS

For the replacement of endogenous losses of nitrogen, the adult rat does not need the following dietary amino acids: lysine, leucine, histidine, arginine, and phenylalanine. These amino acids are all required for growth in the rat.

The adult rat requires both cystine and methionine for the maintenance of nitrogen equilibrium. The cystine requirement can be covered by dietary methionine, but methionine requirement cannot be satisfied by dietary cystine.

The endogenous metabolism of the adult rat involves the destruction of tyrosine, or of tissue constituents derived from tyrosine, but not the destruction of phenylalanine. The

losses thus incurred may be replaced by either dietary tyrosine or by dietary phenylalanine.

The differences between the dietary requirements of amino acids for growth and for maintenance of nitrogen equilibrium may be satisfactorily explained on the basis of differences between rates of supply and of demand with respect to the two functions.

The adult rat may be maintained in nitrogen equilibrium on a nitrogen supply containing only the following amino acids: threonine, isoleucine, tryptophane, valine, methionine, tyrosine and norleucine.

The precise function of norleucine in maintaining the nitrogenous integrity of the tissues needs further study. It may function in promoting the synthesis of lysine or of leucine, or of both.

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THE INTERDEPENDENCE AMONG AMINO ACIDS IN THEIR UTILIZATION IN THE ENDOGENOUS METABOLISM [1]

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There is a clear distinction between the amino acid requirements for the replacement of the endogenous losses of nitrogen and those for the construction of new tissues in growth. In the latter case, the law of the minimum strictly holds: no protein synthesis occurs unless all of the essential amino acids (except arginine) are present in the food supply, while for any given food supply the rate of protein synthesis is determined by the concentration of that essential amino acid present in the smallest proportion with reference to the animal's requirements. However, in the replacement of endogenous losses of nitrogen the law of the minimum does not apply. Incomplete proteins such as gelatin and zein, that are not able to maintain nitrogen equilibrium are nevertheless utilized to a considerable extent (McCollum and Steenbock, '12), and rations containing incomplete amino acid mixtures are definitely superior to those containing no considerable amounts of nitrogen (Mitchell, '16) in the preservation of life. Mitchell and Hamilton ('29) have distinguished these two types of amino acid requirements by the terms "aggregate requirements," referring to the protein synthesis occurring during growth, and "particulate requirements," referring to the maintenance of the nitrogenous integrity of the

[1] The substance of this paper was taken from a thesis submitted by E. Wise Burroughs to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Husbandry, July, 1939.

tissues and to whatever synthetic reactions may be therein involved. The "particulate requirements" of maintenance have been compared to the requirements of inorganic ions (Mitchell, '16).

It has been shown (Burroughs, Burroughs and Mitchell, '40 a) that individual amino acids, particularly isoleucine, histidine, arginine and cystine, are well utilized in the endogenous metabolism, as are also certain incomplete amino acid mixtures. These findings merely confirm similar observations previously reported that have been briefly reviewed recently by Nielsen, Gerber and Corley ('39), all of which support the proposition of the particulate nature of the amino acid requirements for the maintenance of nitrogen equilibrium.

There is, however, other evidence indicating that these requirements are not entirely independent of each other. Elman ('39) found that the injection into dogs of an acid hydrolysate of casein plus added tryptophane and methionine induces nitrogen equilibrium. However, if the tryptophane and methionine are injected 6 hours after the injection of the casein hydrolysate, nitrogen equilibrium does not supervene. The utilization of some of the amino acids in the casein hydrolysate is evidently dependent upon the simultaneous presence in the tissues of tryptophane or methionine or both. Of similar significance are the results of some of the experiments reported in our preceding paper ('40 b). The withdrawal from the daily diet of approximately 4 mg. of nitrogen in the form either of threonine, isoleucine, tryptophane, valine or methionine occasioned average increases in the urinary nitrogen of 37.7, 24.9, 21.2, 24.1 or 16.0 mg. of nitrogen per day, respectively, with no change in the daily intake of nitrogen. Evidently here, also, the utilization in the endogenous metabolism of some amino acids is dependent upon the simultaneous presence of other amino acids in the metabolic mixture.

The experiments to be reported in this paper were designed to throw further light upon this interesting interdependence of one amino acid upon others in replacing the endogenous losses of nitrogen from adult albino rats.

EXPERIMENTAL METHODS

A series of eight amino acid mixtures (table 1) was made up containing from twelve to twenty constituents. In each mixture, the constituent amino acids contributed equal proportions of nitrogen. The mixture of twenty amino acids

TABLE 1

The percentage distribution of nitrogen in the experimental amino acid mixtures

	MIXTURE [1]							
	12	14	16	18	20	14a	16a	18a
dl-threonine	...	7.14	6.25	5.56	5.00	...	...	...
dl-isoleucine	...	7.14	6.25	5.56	5.00	...	...	...
l-tryptophane	...	...	6.25	5.56	5.00	...	...	5.56
dl-valine	...	...	6.25	5.56	5.00	...	...	5.56
dl-methionine	...	...	...	5.56	5.00	...	6.25	5.56
dl-histidine monohydrochloride monohydrate	...	...	...	5.56	5.00	...	6.25	5.56
dl-lysine monohydrochloride	...	...	...	...	5.00	7.14	6.25	5.56
dl-phenylalanine	...	...	...	...	5.00	7.14	6.25	5.56
dl-leucine	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
d-arginine monohydrochloride	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
l-cystine	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
l-tyrosine	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
glycine	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
dl-alanine	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
dl-serine	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
dl-norleucine	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
dl-aspartic acid	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
dl-glutamic acid monohydrate	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
l-proline	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
l-hydroxyproline	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
Total	100	100	100	100	100	100	100	100

[1] For composition of each mixture see text.

was the complete mixture described in our earlier paper ('40 b), while the mixture of twelve acids differed from the complete mixture in not containing the following constituents: threonine, isoleucine, tryptophane, valine, methionine, histidine, lysine and phenylalanine. The intermediate mixtures

were built up by adding to the mixture of twelve acids, pairs of amino acids in the above list, or by subtracting pairs of acids from the complete mixture, according to the following scheme:

Mixture no 12. Contained the complete mixture minus the above eight amino acids.
Mixture no. 14. Mixture no. 12 + threonine and isoleucine.
Mixture no. 16. Mixture no. 14 + valine and tryptophane.
Mixture no. 18. Mixture no. 16 + methionine and histidine.
Mixture no. 20. Mixture no. 18 + lysine and phenylalanine.
Mixture no. 18a. Mixture no. 20 — threonine and isoleucine.
Mixture no. 16a. Mixture no. 18a — valine and tryptophane.
Mixture no. 14a. Mixture no. 16a — methionine and histidine.

The order in which the eight amino acids tested in this way were used was, for the five essential amino acids, roughly in proportion to the size of the negative nitrogen balances induced by their individual withdrawal from the diet. Mixture no. 18 was incomplete, because of the low proportion of tyrosine (table 3, Burroughs, Burroughs and Mitchell, '40 b).

The value of each mixture in the replacement of endogenous losses of nitrogen was determined upon either two or three adult female rats. All mixtures were fed at the same nitrogen level, approximately 80 mg. daily, as supplements to a diet containing only 3.6 mg. of nitrogen, of which each rat received 7 gm. per day. The rats were first placed upon the standardizing diet described elsewhere (Burroughs, Burroughs and Mitchell, '40 b) and then upon the low-nitrogen diet. On the second day on this diet, the amino-acid mixtures were given and were continued until the nitrogen balances were stable. The metabolism apparatus used, the method of feeding, and the methods of collecting, preserving and analyzing the excreta are described elsewhere (Burroughs, Burroughs and Mitchell, '40 b).

DISCUSSION OF EXPERIMENTAL RESULTS

The average data collected in table 2 relate only to those portions of the collection periods for which fairly constant daily nitrogen balances were secured. The collection periods were of 5 to 7 days duration.

The nitrogen balances indicate that the addition of threonine and isoleucine to mixture no. 12 did not improve its value in replacing endogenous losses of nitrogen, although the subsequent addition of valine and tryptophane and then of

TABLE 2

The average nitrogen balance data for the various experimental amino acid mixtures tested

RAT NO.	AVERAGE BODY WEIGHT	AMINO ACID MIXTURE TESTED	DIFFERENCE FROM PRECEDING MIXTURE	NITROGEN METABOLISM PER DAY				
				No. of days averaged	Intake	Urine	Feces	Balance
	gm.			*days*	*mg.*	*mg.*	*mg.*	*mg.*
54	197	12	.....	3	82.6	86.8	13.7	—17.9
55	189	12	.....	5	82.6	96.9	8.5	—22.8
60	168	12	.....	5	82.6	89.1	12.9	—19.4
Aver.	185	12	.....		82.6	90.9	11.7	—20.0
56	190	14	+ threonine + isoleucine	4	82.4	99.1	9.6	—26.3
57	183	14	+ threonine + isoleucine	4	82.4	99.3	10.3	—27.2
50	229	14	+ threonine + isoleucine	4	82.4	98.7	9.9	—26.2
Aver.	201	14	+ threonine + isoleucine		82.4	99.0	9.9	—26.5
58	181	16	+ valine + tryptophane	4	83.2	81.2	12.4	—10.4
59	180	16	+ valine + tryptophane	4	83.2	83.0	9.6	— 9.4
51	219	16	+ valine + tryptophane	4	83.2	84.3	9.2	—10.3
Aver.	193	16	+ valine + tryptophane		83.2	82.8	10.4	—10.0
61	176	18	+ methionine + histidine	4	83.3	86.8	9.8	—13.3
65	157	18	+ methionine + histidine	4	83.3	64.6	28.5[1]	— 9.8
66	218	18	+ methionine + histidine	4	83.3	84.9	11.7	—13.3
Aver.	184	18	+ methionine + histidine		83.3	78.8	16.7	—12.2
52	214	20	+ lysine + phenylalanine	5	83.4	63.6	7.7	+12.1
53	211	20	+ lysine + phenylalanine	5	83.4	64.4	6.8	+12.2
60	172	20	+ lysine + phenylalanine	5	83.4	61.2	11.4	+10.8
Aver.	199	20	+ lysine + phenylalanine		83.4	63.1	8.6	+11.7
71	162	18[1]	— threonine — isoleucine	4	82.9	104.0	10.8	—31.9
72	174	18[1]	— threonine — isoleucine	5	82.9	104.6	9.8	—31.5
Aver.	168	18[1]	— threonine — isoleucine		82.9	104.3	10.3	—31.7
69	165	16[1]	— tryptophane — valine	4	82.6	103.6	7.2	—28.2
70	182	16[1]	— tryptophane — valine	4	82.6	103.4	11.7	—32.4
Aver.	173	16[1]	— tryptophane — valine		82.6	103.5	9.4	—30.3
67	158	14[1]	— methionine — histidine	6	81.4	100.9	9.8	—29.3
68	189	14[1]	— methionine — histidine	6	81.4	108.5	8.5	—35.6
Aver.	173	14[1]	— methionine — histidine		81.4	104.7	9.1	—32.4

[1] Diarrhea.

methionine and histidine decreased the net losses of nitrogen to about half those incurred on mixture no. 12. Furthermore, the withdrawal of threonine and isoleucine from the complete mixture was associated with a marked negative balance of about 32 mg. of nitrogen daily, a level that was not exceeded by subsequent withdrawals first of tryptophane and valine and then of methionine and histidine.

The general picture presented by these balance data indicates that the presence of threonine or isoleucine, or possibly of both, in the diet is necessary for the utilization of the other amino acids essential for nitrogen equilibrium. Also, since the nitrogen balances for rats of the sizes used in these tests (170 to 200 gm.) while subsisting on a nitrogen-free diet would range from about —45 to —55 mg. daily, it appears that some 30 to 50% of these losses can be covered by a variety of incomplete combinations of amino acids, even by mixtures containing none of those acids whose presence in the diet is essential for the attainment of nitrogen equilibrium.

CONCLUSIONS

Some 30 to 50% of the nitrogen lost in the endogenous catabolism may be replaced from a variety of incomplete dietary combinations of amino acids, even by mixtures containing none of those amino acids whose presence in the diet is essential for the attainment of nitrogen equilibrium. This much of the nitrogen requirement for the maintenance of the integrity of the tissues is thus apparently an undifferentiated one, that requires for its complete satisfaction no specific amino acids.

The remainder of the requirement not only relates to specific amino acids, but also is of such a nature that the utilization of the specific amino acids required depends upon the simultaneous presence in the diet of certain combinations of the essential amino acids. Of this interdependence in the requirements of the essential amino acids, it can be concluded, from the experiments reported in this paper, only that threonine and isoleucine, either individually or together, limit the

utilization of the other essential amino acids and possibly occupy a key position in the anabolism consequent upon the endogenous disintegration of tissue constituents.

Thus, the endogenous losses of nitrogen in the adult animal seem to result from the destruction in the tissues of many types of nitrogenous constituents of relatively simple structure, rather than of complex amino-acid aggregates, such as the tissue proteins.

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FACTOR II DEFICIENCY IN DOGS

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Fouts, Helmer, Lepkovsky and Jukes ('38) found that puppies maintained on synthetic casein diet supplemented with vitamin B_1, riboflavin and rice-polish extract containing factor I (vitamin B_6) died with symptoms of blacktongue. Puppies receiving, in addition, purified liver extract containing chick antidermatitis factor (factor II) and nicotinic acid showed normal rate of growth. Although Elvehjem, Madden, Worley and Strong ('37) cured blacktongue by administration of nicotinic acid amide prepared from a similar liver extract, Lepkovsky and Jukes,[1] Dann ('37), Cook, Clarke and Light ('37), and Mickelsen, Waisman and Elvehjem ('38) were unable to cure chick dermatitis by administration of nicotinic acid. It is evident, therefore, that the purified liver extract contains at least two active substances, one of which (nicotinic acid) cures blacktongue in dogs and pellagra in humans, and the other (chick antidermatitis factor) cures pellagra-like symptoms in chicks. The purpose of the present experiment was to study in dogs the deficiency state produced by lack of the factor or factors contained in the purified liver extract other than nicotinic acid.

METHODS

Mongrel adult dogs weighing from 7.1 to 9.4 (average 8.2) kilos were used. Dogs 5, 6 and 7 had been studied during one or more periods of factor I deficiency before the present experiment was started. The synthetic diet, however, had been

[1] Cited as a personal communication in the paper by Fouts, Helmer, Lepkovsky and Jukes ('37).

completely supplemented for at least 1 month before the purified liver extract was excluded from the supplements. The basic diet consisted of the following: washed casein 41.4 gm., sucrose 29.6 gm., Crisco 25.7 gm., bone ash 2.0 gm., and salt mixture no. 185 (McCollum and Simmonds, '18) 1.3 gm. The dogs were allowed to eat as much of the diet as desired. Dogs 1, 2, 3 and 4 received daily by dropper thiamin chloride 50 mcg. per kilo, synthetic riboflavin 60 mcg. per kilo, nicotinic acid 2 mg. per kilo, 0.3 cc. per kilo of rice-polish extract K-85E or 0.6 cc. K-110G per kilo, in addition to vitamins A and D. Dogs 5, 6 and 7 received the same amounts of the above supplements except that 60 mcg. of crystalline factor I per kilo (Lepkovsky, '38) was substituted for the rice-polish extracts. Dogs 5 and 7 received, in addition, 0.5 gm. iron and ammonium citrate and 0.5 mg. copper sulfate daily. The rice-polish extracts containing factor I free from factor II and the purified liver extract (fed to dog 2) were similar to those used previously (Fouts, Helmer, Lepkovsky and Jukes, '38). Red blood cell counts, hemoglobin determinations (Newcomer) and white blood cell counts were made at weekly intervals. Hematocrit measurements were made by use of Wintrobe tubes.

RESULTS

Six of the seven dogs increased in weight after being fed the diet. Dog 3 gained most, namely, 3.7 kilos. Following the preliminary gain in weight, all lost weight (4, 3, 1.9, 5.3, 6, 4, and 4.1 kilos respectively). Loss of weight was associated with progressive decrease in appetite. All animals developed diarrhea within 7 to 66 days; it was intermittent in character but became severe and often bloody before death. Decrease in appetite was especially marked during exacerbations of diarrhea. Vomiting occurred frequently. As weight decreased they became quiet and were very weak shortly before death. The three short-haired dogs (nos. 3, 6 and 7) developed ulcers of the skin over the shoulders, neck and back. The ulcers were superficial, varied in size and number, had a punched-out appearance, bled occasionally, and all showed varying degrees

of healing at time of death of the dogs. Thinning of hair on the head and inner aspects of extremities occurred in dogs 5, 6 and 7. There appeared to be a slight increase in graying of the hair about the mouth in dogs 6 and 7.

The blood findings are presented in tables 1 and 2. Anemia occurred in six of the seven dogs studied. Dog 1 developed a slight anemia by the 252nd day (red blood cell count 5.04 million, hemoglobin 13.4 gm., and hematocrit 38 cc.). Administration of 50 gm. of glucose daily and addition of 6 gm. of sodium chloride to the supplements were followed by a definite but temporary improvement in the general condition and decrease in diarrhea. This improvement was associated with definite hydraemia as demonstrated by a rapid fall in red blood cell count, hemoglobin, hematocrit, blood urea, and serum proteins. At death 36 days later, the red blood cell count had increased to 5.29 million and the hemoglobin to 12.3 gm. Dogs 2 and 4 had more severe anemia than the others (red blood cell counts 4.08 million and 3.67 million, respectively; hemoglobin 11.5 gm. and 8.8 gm.). These dogs did not eat the diet as well as the others and the survival time was shorter. Dog 3 had a slight anemia at the beginning of the studies. This animal gained 3.7 kilos during the first 139 days on the diet. During the same period there was an increase in the red blood cell count and the percentage of hemoglobin. Anemia did not recur although the weight decreased after the 139th day. The erythrocyte counts before death of the three dogs (5, 6 and 7) receiving only the four crystalline supplements were 5.02, 4.87 and 4.93 million, respectively, and the corresponding hemoglobin values 11.0, 14.8 and 12.3 gm. per 100 cc.

Serum proteins decreased in all the dogs examined. There was no increase in serum potassium or consistent fall in plasma chlorides. A terminal rise in blood urea occurred in dogs 4 and 7. The terminal rise in blood urea and decrease in plasma chlorides in dog 7 might have been related to the extensive pneumonia found at autopsy.

Survival on the diet varied between 197 and 289 days (average 243 days) except for two dogs receiving vitamin B_6

TABLE 1

Blood findings in dogs 1, 2, 3 and 4

DOG	DAYS ON DIET	RED BLOOD CELLS	HEMOGLOBIN	WHITE BLOOD CELL COUNTS	HEMATOCRIT	MEAN CORPUSCULAR VOLUME	REMARKS
		millions /cu.mm.	*gm. %*	*per cu.mm.*	*cc./100 cc.*	*cubic μ*	
1	0	7.47	15.3	14,300	51.0	68.3	
	131	6.57	16.3	8,200	50.0	76.1	
	247	5.61	15.6	5,300			
	252	5.04	13.4	5,600	38.0	76.0	
	255	4.35	11.6	17,450	31.0	71.7	
	258	3.59	9.7	15,650	25.0	69.6	
	261	3.43	8.4	5,800			
	272	4.43	11.0	5,100			
	287	5.29	12.3	7,350			
	289						Dead
2	0	7.41	14.3	15,450	47.0	63.4	
	10	7.40	14.2				Purified liver extract (4 cc. per kilo)
	35	5.44	12.5	30,750			
	63	7.41	15.0	11,250			
	65						Purified liver extract discontinued
	112	6.89	15.8	7,350			
	135	4.19	14.4	22,350	31.0	74.0	
	147	4.08	11.5	38,650			
	148						Dead
3	0	5.90	11.9	9,500	41.0	69.5	
	49	6.22	14.9	7,650			
	70	6.14	17.0	7,650			
	77	7.61	18.4	6,600			
	118	5.03	13.9	5,100			
	135	6.27	15.3	7,750	42.5	67.8	
	146	4.80	15.2	8,250			
	168	5.98	18.0	7,450			
	182	6.92	17.2	8,050			
	189	5.74	16.6	8,500			
	196	6.66	18.6				
	197	8.83	24.3	8,050			Dead
4	0	7.51	15.0	12,500	50.0	66.6	
	28	8.20	18.8	30,750			
	49	5.52	11.6	39,450			
	70	4.36	11.7	6,450	31.0	71.1	
	86	3.67	8.8	7,800	26.0	70.8	
	87						Dead

TABLE 2

Blood findings in dogs 5, 6 and 7

TABLE 2

Blood findings in dogs 5, 6 and 7

DOG	DAYS ON DIET	ERYTHROCYTE COUNT IN MILLIONS	HEMOGLOBIN IN GM.%	WHITE CELL COUNT	HEMATOCRIT IN CC./100 CC.	MEAN CORPUSCULAR VOLUME IN CUBIC μ	UREA NITROGEN IN MG.%	TOTAL PROTEIN IN GM.%	ALBUMIN IN GM.%	GLOBULIN IN GM.%	PLASMA CHLORIDE IN MG.%	SERUM POTASSIUM IN MG.%	GLUCOSE IN MG.%	
		per cu.mm.		*per cu.mm.*										
5	—7	5.85	13.3	4,650	40	68.4								
	0	6.45	12.6	5,650										
	84						17.2						56	
	91	6.44	15.8	8,950	42.5	66.4						18.0	57	
	100						15.5	4.70	2.64	2.06	389		67	
	112	5.66	15.0	7,900										
	114						20.0	4.75	2.58	2.17	362		70	
	133	6.05	16.0	7,300	44.0	72.7	32.7	4.59	2.32	2.27	369		93	
	142						25.9	4.76	1.96	2.80	377		103	
	149						26.2	4.70	2.04	2.66	381		100	
	154	4.86	16.8											
	156						13.0	3.86	1.64	2.22	372		111	
	170						11.5	4.00	1.91	2.09	356		104	
	175	5.87	13.1	10,400	36.0	61.3								
	177						12.9	4.34	1.89	2.45	351		90	
	198	5.08	13.6	7,500	36.0	70.8	17.8	4.35	1.66	2.69	358		100	
	205						15.7	4.30	1.61	2.69	354		120	
	212	4.76	11.1		33.0	69.3	14.2	4.48	1.86	2.62	350		144	
	217	5.02	11.0	13,150										
	219						12.1	3.98	2.18	1.80		18.0	118	
	221													Dead
6	0	7.44	16.8	7,950										
	93	6.96	15.5	3,450	44.0	63.2								
	204						14.3						61	
	209	6.16	15.3	5,550	44.0	73.8						21.0	53	
	218						19.8	5.75	3.12	2.63	383		63	
	224	6.36	17.0	5,100										
	225						13.5	5.74	3.36	2.38	379		68	
	230	6.37	16.5	5,350			10.0	5.94	3.03	2.91	382		59	
	251	4.47	14.9	33,950	35.0	78.3	18.5	5.11	2.63	2.48	329		114	
	252	4.62	12.8	40,200	33.5	72.5						22.0		
	257	4.87	14.8	4,300										Dead
7	—8	7.61	14.8	8,800	47.0	61.8								
	0	7.78	16.6	5,150										
	93	8.17	18.4	7,900	51.0	62.4								
	204						17.2						43	
	209	6.60	15.8	9,000	46.0	69.7						15.3	46	
	218						12.7	4.98	2.36	2.62	369		63	
	224	5.89	14.5	29,000										
	225						12.8	5.45	2.55	2.90	390		45	
	230	5.54	13.4	16,400										
	232						16.4	5.50	2.38	3.12	387		48	
	250	4.93	12.3	5,500			157.0	4.91	1.89	3.02	317		83	Dead

supplied as a component of the rice-polish extract. These dogs died on the eighty-third and eighty-seventh day. They showed loss of appetite within 4 to 7 days. One animal (dog 2) stopped eating and developed a diarrhea on the seventh day. Factor II (liver extract CF I, 4 cc. per kilo) was added to the supplements given from the tenth to the sixty-third day. All symptoms slowly disappeared and body weight gradually increased. When the liver extract was discontinued the symptoms recurred within 47 days and the dog died within 83 days. The other animal ate very little from the start, its food intake averaging 47 gm. per day. Survival of the three dogs receiving the four crystalline supplements varied between 221 and 257 days.

At autopsy the liver of each dog appeared to be fatty. One animal had superficial ulcerations of the gums, another had numerous shallow ulcers and hemorrhages in the ileum, and dog 6 had a penetrating ulcer on the lesser curvature of the stomach as well as numerous superficial ulcers scattered throughout this organ and the first portion of the duodenum. The bone marrow of dogs surviving for long periods of time showed increased cellularity. Dogs 2 and 7 had extensive pneumonia.

DISCUSSION

The deficiency disease which developed in dogs subsisting upon a synthetic casein diet supplemented with thiamin chloride, synthetic riboflavin, nicotinic acid, and rice-polish extract containing factor I or crystalline factor I, was similar to the deficiency state produced by Chick, Macrae, Martin and Martin ('38) in pigs fed a synthetic diet similarly supplemented. Central nervous system involvement observed in factor II deficiency by Chick, Macrae, Martin and Martin ('38) and by Wintrobe, Mitchell and Kolb ('38) in pigs, and by Phillips and Engel ('39) in chicks, was not observed.

The anemia noted in six of the seven dogs was similar to that found by Chick, Macrae, Martin and Martin ('38) and Wintrobe, Sawter and Lesco ('39) in swine. It was of moderate severity and there tended to be an increase in mean cor-

puscular volume. The decrease in serum proteins which occurred in all the dogs examined might account for this tendency (Bethell, '36). The two dogs which ate the least amounts of the diet had the most severe anemia at death. Study of blood electrolytes revealed no evidence suggesting adrenal insufficiency. These findings do not substantiate those of Morgan and Simms ('39) who found striking and constant atrophy of adrenals in rats deprived of filtrate factor or factors. The ulcers of the skin in the short-haired dogs, however, are similar to those reported by these authors.

There appeared to be no difference in the symptoms of the deficiency states in dogs receiving the rice-polish extract and those receiving the crystalline factor I except that two of the dogs receiving the rice-polish extract succumbed in a shorter time. The survival time of the other five dogs was fairly constant. It would appear that the previous dietary history must have influenced the time of development of symptoms and survival time. Dog 2 developed symptoms within 7 days when first started on the deficient diet but did not show any symptoms for 47 days after having received purified liver extract for 53 days. The three dogs receiving similar amounts of purified liver extract for long periods of time (163 to 619 days) previous to this experiment survived from 221 to 257 days. Coprophagy probably influenced the survival time but it did not appear to be the determining factor inasmuch as when it occurred it was not observed until late in the study and after symptoms of the deficiency had developed.

SUMMARY

1. Adult dogs fed a synthetic casein diet, supplemented with thiamin chloride, riboflavin, nicotinic acid, and crystalline factor I (vitamin B_6), and apparently deficient only in a factor or factors contained in a purified liver extract other than nicotinic acid, developed a deficiency state characterized by loss of appetite, marked loss of weight, intermittent diarrhea, moderate anemia, and death.

2. Short-haired dogs showed ulcerations of the skin while long- or wire-haired dogs did not.

3. It was not determined whether the deficiency state described is due to lack of factor II (chick antidermatitis factor) alone or to the lack of this and other as yet unisolated components of the vitamin B complex.

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THE RETENTION OF CALCIUM AND PHOSPHORUS BY PRE-SCHOOL CHILDREN [1]

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ONE FIGURE

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The feasibility of supplementing the diets of children with certain vitamins and minerals has been demonstrated by Aykroyd and Krishnan ('38), Privitera ('38), Schlutz and his associates ('38), Summerfeldt and Ross ('38), and Tisdall et al. ('30). From past experience it is known that the utilization of minerals is influenced by the source. Thus, the question arises as to the availability of added substances in fortified foods for use by the human body.

Several studies of the calcium metabolism of children have been made in recent years (Daniels et al., '34, '35; Outhouse et al., '39; Kinsman and associates, '39). A survey of the available data shows that marked variations in calcium retention have been noted. Furthermore, individual retentions differ materially from time to time in the same child. Fairbanks and Mitchell ('36) have shown with growing rats that calcium requirements can be determined only when the body stores have been saturated by appropriate pre-feeding.

Several investigations comparing the availability of calcium and phosphorus from milk and from inorganic sources have

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[2] The data on phosphorus metabolism were presented by Mr. Meservey in partial fulfillment of the requirements for the degree of master of science, University of Vermont, June, 1939.

been made. A study of these data, along with their own, led Henry and Kon ('39) to make the following statement, "As far as they go, these studies appear to indicate that if there is a difference between the availabilities of calcium from milk and from inorganic sources it cannot be very marked and our metabolic experiments confirm such a view."

The object of this study has been to determine whether the calcium of an alkaline salt mixture added to a wheat farina would be as well utilized by pre-school children as an equivalent amount of this element supplied by milk. After adequate pre-feeding to insure saturation with calcium and phosphorus, part of the milk of the basal diet was omitted, and the calcium in this quantity of milk was replaced by an equivalent amount furnished by the salt mixture[3] in the fortified cereal.

EXPERIMENTAL

Ten children, six boys and four girls, whose ages, weights, and heights at the beginning and end of the experiment are shown in table 1, were selected to serve as subjects. A routine was established to make a program as nearly normal as possible within the rigid control required. In inclement weather, the children received exposure to rays from an ultraviolet lamp for 15 minutes daily.

The diets were similar to those used by Daniels et al. ('34). A typical diet for one subject throughout the study is illustrated in table 2. The basal diet was planned so that approximately 50 mg. of calcium per kilogram body weight per day or 80% of the total ingested was supplied by milk and the remainder by the solid food. During experimental periods, 200 mg. of milk calcium were replaced by 200 mg. of calcium in the fortified cereal. The food was supplemented with 1800 U.S.P. units of vitamin D, 12,000 U.S.P. units of vitamin A, and 300 U.S.P. units of thiamin-hydrochloride daily. Distilled water was used for drinking and cooking.

The children were divided into two groups as evenly matched as possible with regard to weight and age. These two groups

[3] A mixture of calcium, sodium and iron phosphates.

TABLE 1
Growth record of children

Subject	Group	Sex	BEGINNING			END						
			Age	Weight	Height	Age	Weight	Height	Gain in weight	Gain in height	Expected gain [1]	
											Weight	Height
			yrs. mos.	*kg.*	*cm.*	*yrs. mos.*	*kg.*	*cm.*	*kg.*	*cm.*	*kg.*	*cm.*
1	LP	F	5 – 10	16.3	102.2	6 – 4	18.5	105.4	2.2	3.2		
2	LP	F	4 – 2	16.2	101.0	4 – 8	18.4	105.4	2.0	4.4	1.1	4.1
3	PL	M	5 – 0	18.5	102.9	5 – 6	20.3	108.6	1.8	5.7	1.1	3.4
4	PL	M	5 – 4	18.0	106.7	5 – 10	20.4	111.1	2.4	4.4	0.7	3.5
5	PL	F	2 – 11	14.5	95.3	3 – 5	17.0	98.4	2.5	3.1	0.2	2.5
6	LP	M	3 – 7	17.6	109.5	4 – 1	19.6	113.7	2.0	4.2	1.0	3.6
7	LP	M	5 – 5	17.7	99.7	5 – 11	19.1	104.8	1.4	5.1	0.5	2.6
8	LP	M	5 – 4	20.1	114.3	5 – 10	22.9	120.0	2.8	5.7	0.7	2.6
9	PL	F	4 – 2	15.5	99.1	4 – 8	17.3	103.5	1.8	4.4	1.1	4.1
10	PL	M	4 – 0	14.9	94.6	4 – 6	17.0	97.8	2.1	3.2	0.9	3.5

[1] Based on data at Iowa Child Research Station ('29).

TABLE 2

Sample diets. Child no. 2 in group 1. Figures are in grams or cubic centimeters unless noted differently

	PERIOD 1	PERIOD 2	PERIOD 3	PERIOD 4	PERIOD 5
Breakfast					
Oatmeal	10	10	10	10	—
Cream of Wheat	—	—	—	—	15 New
Sugar	7	7	9	7	7
Milk	225	225	162	225	225
Bread	15	15	15	15	15
Butter	5	5	5	5	8
Jelly	10	10	10	10	10
Prunes	40	40	40	40	40
Egg	50	50	50	50	50
Mid A.M.					
Orange juice	120	120	120	120	120
Vit. B tablets	1 tablet	1 tablet	1 tablet	1 tablet	1 tablet
Oleum Percо. (vit. D)	10 drops	10 drops	10 drops	10 drops	10 drops
Dinner					
Cream of Wheat	—	—	—	—	10 New
Beef	30	30	30	30	30
Potato	85	85	85	85	85
Beans	40	—	—	40	—
Carrots	—	40	40	—	40
Bread	15	15	15	15	15
Butter	10	10	10	10	10
Milk	20	20	20	20	20
Tomato juice	110	110	110	110	110
Doughnut	—	—	23	—	—
Mid P.M.					
Banana	70	70	70	70	70
Milk	218	218	—	218	50
Brazil nuts	—	—	14	—	—
Supper					
Cream of Wheat	15 Old	15 Old	15 Old	15 Old	15 New
Sugar	7	7	9	7	7
Milk	225	225	162	225	225
Bread	15	15	15	15	15
Butter	5	5	5	5	9
Jelly	10	10	10	10	10
Apple sauce	60	60	60	60	60
Chocolate	15	15	15	15	15
Ca lactate	2.856*	—	—	—	—
Triple PO_4	—	—	—	1.213*	—

* Equivalent to 256 mg. calcium.

are designated as LP and PL, respectively: the former being saturated with calcium from lactate in the first and calcium from phosphate in the second saturation period. PL children were saturated with calcium from phosphate in the first and calcium from lactate in the second saturation period. The experiment was divided into five metabolism periods as shown in table 3.

TABLE 3

Diet periods

GROUP	PERIOD 1 6 WEEKS	PERIOD 2 5 WEEKS	PERIOD 3 2 WEEKS	PERIOD 4 4 WEEKS	PERIOD 5 6 WEEKS
LP	Basal diet plus 200–256 mg. Ca from lactate	Basal diet	Ca intake reduced to approximately ⅔ that of basal diet	Basal diet plus 256 mg. Ca from triple phosphate	Basal diet except 200 mg. milk Ca replaced by 200 mg. Ca supplied by fortified farina
PL	Basal diet plus 200–256 mg. Ca from triple phosphate	Basal diet except 200 mg. milk Ca replaced by 200 mg. Ca supplied by fortified farina	Ca intake reduced to approximately ⅔ that of basal diet	Basal diet plus 256 mg. Ca from lactate	Basal diet

To rule out variations which might be assigned to changes of weather, season and age, the periods for the PL group were reversed and the sequence was 4, 5, 3, 1, 2.

The dietary routine was interrupted for a few days between periods, care being taken that the calcium intake remained the same as that of the preceding period.

DISCUSSION OF RESULTS

Calcium. By referring to table 4 and figure 1, it may be seen that retention during the first two periods was higher than that noted during the last three. It has been noted in other studies of this nature that previous depletion due to inadequate mineral intake or poor utilization may be responsible for early high retentions. In period 1 children (LP), being saturated with the lactate, retained more calcium than those obtaining this element from the phosphate mixture. Using the data for

TABLE 4

Calcium and phosphorus metabolism

PERIOD	GROUP	CALCIUM							PHOSPHORUS						Ca : P [1]	Ca : P FOOD	WEEKLY STOOL WEIGHT	NITROGEN RETENTION PER KG. PER DAY
		Weekly intake	Weekly output			Weekly retention		Ca per gram, stool	Weekly intake	Weekly output			Weekly retention					
			Amount	Feces	Urine					Amount	Feces	Urine						
		gm.	*gm.*	%	%	*gm.*	%	*mg.*	*gm.*	*gm.*	%	%	*gm.*	%			*gm.*	*mg.*
1	LP [2]	9.19	7.08	95.9	4.1	2.11	22.9	10.1	8.72	7.37	42.7	57.3	1.35	15.5	1.58	1.05	682	
1	PL [3]	9.17	7.57	95.2	4.8	1.60	17.4	11.1	9.80	8.52	38.2	61.8	1.28	13.2	1.21	0.94	657	
2	LP	8.14	6.43	93.3	6.7	1.71	21.1	10.1	8.46	7.63	44.6	55.4	0.83	10.4	2.04	0.96	580	61.2
2	PL	7.90	5.99	91.5	8.5	1.91	24.1	9.7	8.43	7.33	35.5	64.5	1.10	13.1	1.75	0.94	571	53.6
3	LP	5.01	4.06	90.0	10.0	0.95	18.9	6.8	7.33	6.68	43.4	56.6	0.65	8.8	1.66	0.68	542	
3	PL	4.73	3.90	88.2	11.8	0.83	17.7	6.4	7.06	6.50	39.7	60.3	0.56	8.1	1.48	0.67	547	
4	LP [2]	9.69	8.24	95.0	5.0	1.45	15.0	12.2	9.94	9.29	48.1	51.9	0.65	6.8	2.18	0.97	640	55.0
4	PL [2]	9.32	7.91	92.7	7.3	1.41	15.1	10.6	8.16	7.49	38.2	61.8	0.67	8.6	2.06	1.14	692	65.0
5	LP	8.12	6.62	93.3	6.7	1.50	18.5	11.0	8.80	7.78	43.0	57.0	1.02	11.5	1.49	0.92	563	51.9
5	PL	7.74	6.29	91.8	8.2	1.45	18.7	10.3	8.41	7.54	36.5	63.5	0.89	10.4	1.65	0.92	566	53.1
Av.	LP	8.03	6.49	93.5	6.5	1.54	19.3	10.9 [4]	8.64	7.75	44.4	55.6	0.90	10.6	1.79	0.92	616	56.0
Av.	PL	7.77	6.33	91.9	8.1	1.44	18.6	10.4 [4]	8.37	7.48	37.6	62.4	0.90	10.7	1.63	0.92	621	57.2

[1] Calculated on basis of milligrams retained per kilogram body weight per day.

[2] Calcium lactate used for saturation.

[3] Tri-calcium phosphate used for saturation.

[4] Data of period 3 omitted in computing average.

Wet ashing urine, feces and foods: Gerritz ('35).

Calcium: Shohl and Pedley's ('22) modification of McCrudden's ('09–'10; '11–'12) method.

Phosphorus: Mackay and Butler's modification of Mathison's method, Peters and Van Slyke ('32).

Total nitrogen: Kjeldahl method.

period 2 as a base, an equivalent of all of the calcium added and more was excreted in period 1 by both groups. Since amounts of calcium equivalent or more than equivalent to those added were excreted in the period of saturation, as compared with those where basal diets alone were used, it may be assumed that the basal diets contained adequate calcium for the needs of these children. In period 2, when the calcium intake was reduced and a comparison made of the availability of calcium derived from milk and from the fortified cereal, the latter source proved to be the better one from the standpoint of retention.

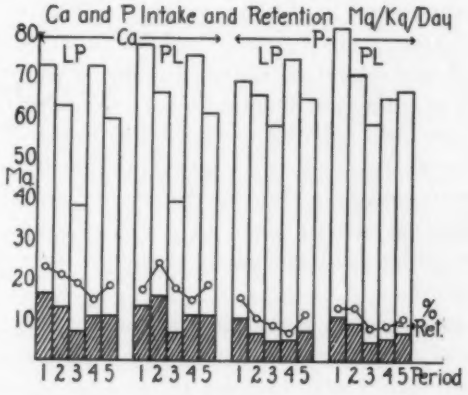

Figure 1

In period 3 the calcium-phosphorus ratio of the food, instead of being approximately 1 as in other parts of the study, approached a value of 0.7. This in itself may offer an explanation of the lowered retention. The percentage of calcium excreted in the stools, however, reached its lowest point suggesting that the disproportionate amount of phosphorus in the diet had not caused any unusual precipitation of calcium in the intestine. An adequate intake of vitamin D, however, tends to eliminate unfavorable effects due to low calcium-phosphorus ratios, and our diets contained plenty of this vitamin. Further, Sherman and Hawley ('22), using diets with low calcium-phosphorus

ratios over short periods of time, obtained data which indicated no disturbance of calcium retention.

It is interesting to note that the calcium intakes and retentions in period 3 of this study are in close agreement with those of Outhouse and her associates ('39). In the last two periods with higher intakes, the amounts retained are similar to those found by Sherman and Hawley ('22), while in the first two periods the retentions are higher. The maximum retentions did not approach those reported by Stearns and Jeans ('34). To obtain maximum retentions under the conditions of this experiment, the amount of calcium in the diet had to be greater than that suggested by Outhouse and associates ('39), but these investigators have pointed out that their data may not apply to all pre-school children. In regions of the country where there is little sunshine and where the outdoor activities of children are limited by the severity and length of the winter, it may be necessary to furnish more calcium than in regions having a more moderate climate. From our results one would conclude that the old standard of 1 gm. of calcium per day would more nearly fulfil the needs of the pre-school child than smaller amounts.

The retentions were higher in the fourth than in the preceding period and there was no significant difference between the results obtained with the two salts. Less calcium was retained by each group than in the first period. Increasing the amount of calcium resulted in a better retention of this element, but not of phosphorus (Tisdall and Drake, '38). In period 5 retentions were the same regardless of the source, showing that calcium of the fortified cereal was just as satisfactory in fulfilling the needs of the pre-school child as an equivalent amount from milk. This satisfactory utilization of calcium from an added salt is in agreement with that found by Stearns and Jeans ('34). Since the salt mixture used for fortification contained disodium phosphate, it is of interest to note that Henry and Kon ('39) found that the addition of this salt to a milk diet increased the absolute retention of calcium

and phosphorus by rats. The calcium-phosphorus ratios of food ingested approximated 1:1 as in our diets.

If the calcium output during period 5 is compared with that of period 4, it is seen that the differences between the total excretions correspond almost exactly with the extra amounts added for saturation. This affords further confirmation of our previous assumption that the basal diets were adequate in their calcium content. Since the retentions by both groups of children were essentially the same in periods 4 and 5, it is evident the children were saturated with this element.

Statistical treatment of the data for calcium retention reveals no significant differences between the two groups except in period 2. During this time, children receiving the diet in which part of the milk calcium had been replaced by calcium from the fortified cereal retained more calcium than members of the other group. Although the retentions in period 1 appeared to favor lactate as a better source of calcium than phosphate, the observed difference proved to be insignificant.

Phosphorus. The amounts stored by the children were larger at the beginning than toward the end of this investigation. This raises the question, as with calcium, as to the effect of previous depletion upon the extent of storage. The presence of more phosphorus in the diet of the PL than in that of the LP children did not increase the retention of the former group. This observation confirms that of Wang and her associates ('29) who found that increasing the amount of phosphorus in the diet over and above requirements merely caused a larger excretion in urine and feces. During the first period, when PL children were being saturated by use of the phosphate, about 12% of the phosphorus of the added salt was retained. Quite a different result was obtained with the other children (LP) while they were being saturated with the phosphate in period 4, for none of the added phosphate phosphorus was retained; in fact an equivalent of all of the added phosphorus and more was excreted during this period. Accordingly, it may be assumed that the basal diets contained sufficient phosphorus to meet the requirements of these children. The amounts re-

tained in period 1 were alike for each group, showing that the quantity added had no effect upon retention. The data for period 2 show a better retention of phosphorus when part of the milk was replaced by fortified cereal. Admittedly, the intake per kilogram body weight per day was higher for PL children who retained the larger amount of phosphorus. Yet, with a more marked difference in the daily intake per unit of body weight in the first period, no such difference in retention was found.

The girls in each group had a lower percentage output of phosphorus in the urine than did the boys. The question may be raised as to whether this finding might be due to the urinary contamination of the feces by the girls. Extreme care was used in an effort to avoid this, and the girls always were required to empty the bladder before going to stool. The nurse in charge then watched the pails to see that no urine had been passed.

Stearns ('31) has discussed the significance of the retention ratios of calcium and phosphorus in children. A survey of the literature dealing with the calcium and phosphorus metabolism of children reveals that the retention ratios vary within wide limits over a period of time even with the same child. Our data confirm this observation, but ratios obtained by us are much higher than those listed by Stearns ('31). Our average retention ratios during 6 months were 1.79 for LP and 1.63 for PL children. These values lie within the range, namely, between 1.5:1 and 2:1, recommended by Stearns.

The calcium-phosphorus ratios of bones of children as determined by several workers show some degree of variation, the values ranging from 1.9 to 2.26. In computing the amount of phosphorus required for converting retained calcium into bone, the selection of the proper ratio is important if a correct estimate is to be made of the amount of phosphorus remaining for synthesis of soft tissues. If we accept the higher ratio of 2.26 as correct, then with any ratio lower than this, phosphorus would be available for building soft tissue. We could conclude then that at all times during this study each group was building bone and soft tissue. Our data tend to confirm the idea that

in given periods some tissues grow at a more rapid rate than others, thus requiring different amounts of phosphorus. It is interesting to point out that during period 4 when high retention ratios were found, the weekly increase in body weight of children of both groups was smaller than in any other period.

The average daily retentions of phosphorus in milligrams per kilogram body weight for the 6 months were 6.9 for the LP and 7.4 for PL children. Both of these values are slightly lower than the average daily value of 8 mg. per kilogram body weight obtained by Sherman and Hawley ('22). The average retentions found by Daniels et al. ('35) also are somewhat higher than those obtained in this study.

As with calcium, statistical treatment of the data showed no significant differences between the amounts of phosphorus stored by the two groups except in period 2. Here children (PL) on the diet in which part of the milk calcium had been replaced by calcium from the fortified cereal retained significantly more phosphorus than the other children.

Nitrogen balances were determined for 1 week in periods 2, 4, and 5, and all children were in positive balance (table 4). The highest and lowest retentions found during period 2 amounted to 93 and 41 mg. per kilogram body weight per day respectively; in period 4, to 92 and 22 mg.; and in period 5, to 64 and 37 mg. The few values obtained revealed that no one child consistently retained a large or a small amount of nitrogen. In general the retentions were somewhat lower than those found by Daniels et al. ('35).

By referring to data in table 4 it is seen that there is no evidence of any laxative effect of the salt mixture used for fortification. The average weekly weights of stools of the two groups are in close agreement throughout the study with the possible exception of period 4, in which a higher value was noted with children who were receiving calcium lactate.

To gain information as to the possible effects of the triple phosphate mixture upon acid-base balance, titratable acidity (Folin, '05) of the urine was determined during period 5. Here again there was no significant difference for the two groups,

the average daily titration expressed in cubic centimeters of N/10 alkali being 156 for the LP and 168 for the PL group. These values are in agreement with those of Hawks et al. ('37).

SUMMARY AND CONCLUSIONS

A comparison has been made between milk and a fortified cereal as sources of calcium and phosphorus for pre-school children. The subjects were four girls and six boys ranging in age from 3 to 6 years. To rule out possible variations which might be due to changes in age, weather and season, the children were divided into two groups each consisting of two girls and three boys and the sequence of diet periods was reversed.

An adequate basal diet was used. To eliminate effects of previous calcium depletion, if any, extra calcium for saturation was fed in the form of lactate or the phosphate mixture used for fortification of the cereal. Final analysis shows that there is no significant difference between the retentions of calcium and phosphorus regardless of the salt used.

With the same basal diet the retention of calcium and phosphorus was determined when 200 mg. of milk calcium was replaced by 200 mg. of calcium in the fortified cereal. In one period the storage of calcium and phosphorus was significantly better when these two elements were supplied in part by the fortified cereal rather than by milk, while in a second period of comparison the availability of calcium and phosphorus as measured by retention was the same.

The children were in positive calcium, phosphorus and nitrogen balance throughout the study and more phosphorus was stored than was required to convert retained calcium into bone.

The averages of the results obtained for two groups of children over a period of 6 months were in close agreement. The daily storage of calcium was between 11 and 12 mg. and of phosphorus between 7 and 8 mg. per kilogram body weight. About 19% of the calcium and 11% of the phosphorus were retained. All children grew at rates better than those given as normal by the Iowa Child Research Station.

For maximal retention of calcium it was necessary to have a daily intake of over 700 mg. of calcium. When a part of the milk calcium of an adequate diet was replaced by an equivalent amount supplied by tri-calcium phosphate in a fortified cereal, utilization as measured by retention was equally good from milk and cereal.

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